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CHEMISTRY OF THE METABOLITES OF *STEREUM PURPUREUM*

by

© MOHAMMAD HOSSEIN SAEEDI-GHOMI

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

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CHEMISTRY OF THE METABOLITES OF STEREUM PURPUREUM

submitted by MOHAMMAD HOSSEIN SAEEDI-GHOMI

in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Chemistry.

Supervisor - W.A. Ayer

External Examiner

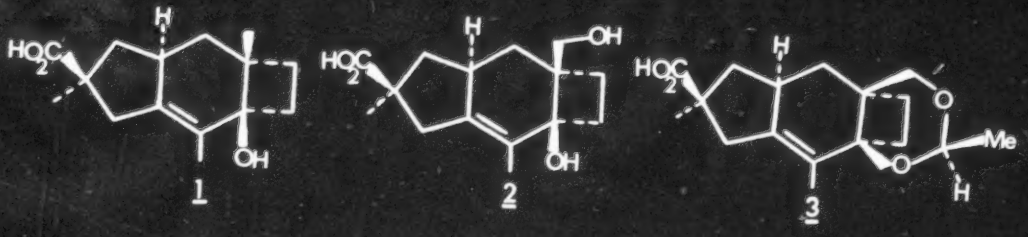
Date Dec. 12, 1980

تقدیم به :

زیبا ، خانواده‌ام ، و مادر که نماند

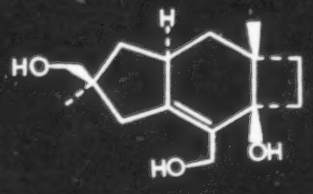
ABSTRACT

Stereum purpureum is the fungus responsible for silver leaf disease in a variety of fruit trees, especially plum and apple. Part I of the thesis describes an investigation of the metabolites produced by this fungus when grown in liquid culture. Sterpuric acid (1), the first compound isolated from the acidic fraction of the culture broth extract, represents a completely new skeleton among the sesquiterpenoids. Its structure was established by chemical transformations and physical methods. Further examination of the acidic fraction afforded two other metabolites with the carbon skeleton of sterpuric acid, hydroxysterpuric acid (2), and hydroxysterpuric acid ethylidene acetal (3). The two latter compounds were isolated in the form of their methyl esters.



Examination of the neutral fraction of the broth culture extract yielded sterpurene-3,12,14-triol (4), which also possesses the sterpuric acid skeleton. The neutral fraction also contains two new compounds, 5

and 6 (temporarily called lactone "A" and lactone "B"), which have the carbon skeleton of isolactarane.



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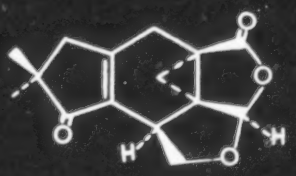

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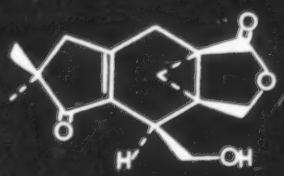

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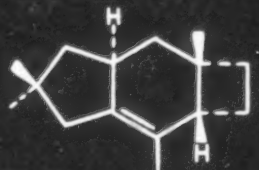
The last compound discussed in Part I of the thesis is the sesquiterpene hydrocarbon sterpurene (7). This compound was isolated from the mycelium of S. purpureum.



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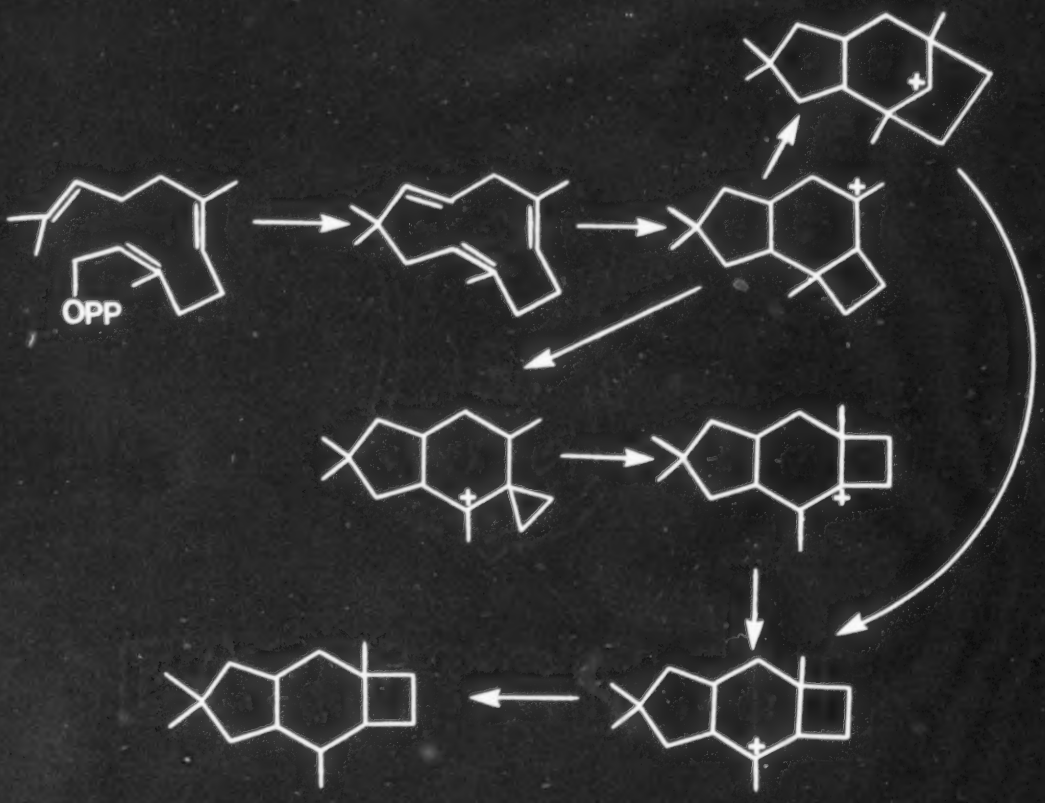
The second part of the thesis describes a study concerning the biosynthetic pathway leading to the sterpurane skeleton. S. purpureum cultures were grown in the presence of either [1-^{13}C]- or [2-^{13}C] acetate. Examination of the ^{13}C nmr spectra of the derivatives of sterpuric acid and sterpurene-3,12,14-triol obtained from these experiments indicated that the sterpurane skeleton is biosynthesized as shown in the following scheme.


OPP

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I: METABOLITES OF THE FUNGUS STEREUM PURPUREUM

INTRODUCTION

Stereum purpureum (Pers.) Fr. has been known to man for nearly a century.[1] This fungus has been classified[2] as belonging to the class Basidiomycetes, subclass Homobasidiomycetes, order Aphyllophorales, family Thelephoraceae and genus Stereum. It is now well established that this fungus is responsible for a disease in a variety of fruit trees, especially plum, apple, and peach, in which silvering of the foliage is its common symptom. The first observation on this disease was made by a French scientist in 1885 but it was soon known throughout Europe particularly in England, North America, Australia and New Zealand.[3]

The name "silver leaf" was first applied to the disease by Percival[4] in 1902 who was the first to question the non-parasitic and "Acts of God" character of the disease. He showed that hyphae of the fungus were present in the wood and root of silvered trees, and by inoculation proved that S. purpureum could cause the silvering.

The results of Percival were fully substantiated by Brooks in a series of studies in England.[5-8] He carried out extensive inoculation experiments on plum and apple trees and proposed the mechanism of action of S. purpureum in causing silver leaf disease which is currently the accepted mechanism.[9,10]

According to his studies the fungus enters the

wood through pruning wounds and broken branches, frequently it being drawn a considerable distance into the vessels where it germinates under more favorable conditions chiefly because of the abundance of carbohydrates in these tissues. It then causes girdling and dieback. The silvering of the leaves is a secondary symptom caused by toxic materials of the fungus which are carried up by the transpiration current. Brooks successfully demonstrated this by causing silveriness in plum leaves by injection of a filtered extract of the fungus into the stem.

The silveriness of the foliage is primarily due to partial separation of the mesophyl cells from one another and from the epidermis. Owing to the presence of abnormal air spaces thus induced, the light reflecting qualities of the leaf are altered, and the leaves appear silvery. At a rather late stage in the development of the disease and often as the affected trees or branches die, numerous bracket-like fruit bodies appear which vary in size from about 1/4 to 2 in. across. The upper surface of the fructification is yellowish-brown, hairy and zoned, the lower surface smooth and purplish. This lower region carries numerous spores and it is by means of these microscopic bodies that the disease is spread.[11]

In 1909, Güssow detected the first authentic case

of silver leaf disease in Canada.[12] He discovered the existence of S. purpureum on a diseased apple tree in the orchards of the Truro Agricultural College in Nova Scotia. Further investigation by this scientist revealed the widespread existence of silver leaf disease on plum and apple trees from Nova Scotia to British Columbia. In his publication, Güssow also took the opportunity to draw the attention of his United States colleagues to the existence of the disease in North America despite the lack of previous reference to its occurrence on this side of Atlantic. However, silver leaf disease was not detected in the United States until 1923,[13] but, it occurs widely on apple and plum trees in the East, the West and the Lake states.[14] In the case of the province of Alberta this disease is presently found on mountain ash, cotoneaster, and aspen.[15]

Beever[16] succeeded in growing S. purpureum in liquid medium. This author undertook a study to determine the ability and extent to which this fungus is able to use a range of nutrients for mycelial growth in vitro. A freshly isolated culture of S. purpureum maintained on potato dextrose agar (PDA) or 1.5% malt agar was used throughout. A defined liquid medium containing glucose as carbon source and L-glutamic acid as nitrogen source in which this fungus produced good

growth was selected as the basal medium, and the suitability of other carbon and nitrogen sources was determined by substitution. The influence of several vitamins and of calcium on growth was also investigated. Twenty-three carbon compounds and twenty-seven nitrogen-containing compounds were tested by adding each one separately to the basal medium to provide either 10 g per liter of carbon, in place of glucose or 225 mg per liter of nitrogen, in place of glutamic acid. These experiments showed that maximum mycelial growth occurred with mannose or soluble starch as carbon source and with glutamic acid or glutamine as the nitrogen source after a growth period of 30 days. Of the seven vitamins tested only thiamine was required for growth and the addition of calcium hydrogen phosphate to the medium resulted in an increase in the yield of mycelium, although considerable growth occurred in its absence.

Recently, there have been reports which claim that toxic proteins of S. purpureum are causative of silver leaf symptoms on apple trees.[17,18] S. purpureum was cultured at 30 degrees for 20 days on a potato extract medium. The culture filtrate was dialized against 0.005 M phosphate buffer (pH 6.0) and then the dializate was mixed with solid ammonium sulfate to 90% saturization. The resulting precipitate was dialized against

0.02 M phosphate buffer (pH 6.2) and passed through a column of DEAE-cellulose and the active substance was precipitated with ammonium sulfate followed by column chromatography on sephadex G-100. The active substance thus isolated had a molecular weight of 50,000 and induced silver leaf symptoms when injected into apple trees at a dosage of about 25 µg per tree .

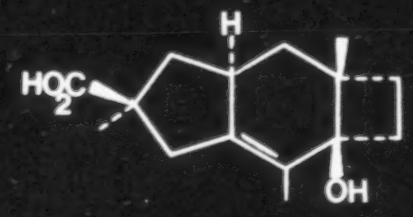
Since it seemed well established that the silver leaf disease is caused by the action of a metabolite or metabolites produced by S. purpureum and since to the best of the author's knowledge there has been only one attempt reported[19] for the isolation of metabolites of the mycelium of this fungus in which ergostrol as well as an unidentified alcohol with molecular ion peak (M^+) of 398 and an unidentified aliphatic ester were isolated, it seemed appropriate to undertake a systematic investigation concerning the isolation of metabolites of this fungus.

The purpose of the work reported in thesis was to separate and characterize the metabolites produced when S. purpureum is grown in liquid culture and to determine the structure of these metabolites.

When S. purpureum is grown in liquid culture its broth extract contains many metabolites, the vast majority of which are sesquiterpenoids. Out of seven sesquiterpenoid metabolites listed in the chart only

one of them (5) was isolated from the mycelium. It should be pointed out that compounds 2 and 3 were isolated in the form of their methyl esters. Furthermore, compounds 1-5 represent a new structural type among the sesquiterpenoids. Thus, the name sterpuric acids and sterpurenes are used for 1-3 and 4-5 respectively. Compounds 6 and 7 have the carbon skeleton of the known compound isolactarorufin.[20] All compounds were identified by spectral analysis and chemical transformations. The structure of sterpuric acid 1 which is deduced herein on the basis of chemical and spectroscopic studies has been confirmed by a single crystal x-ray diffraction analysis performed by Professor Jon Clardy and his group at Cornell University.

The final chapter of this thesis will be concerned with the determination of the biosynthetic pathway leading to the sterpurane skeleton by utilization of Carbon-13 nuclear magnetic resonance (^{13}C nmr) studies of the compounds produced when the liquid culture was fed with [1-^{13}C]- and [2-^{13}C]-acetate. The introduction to biosynthetic studies will appear accordingly.



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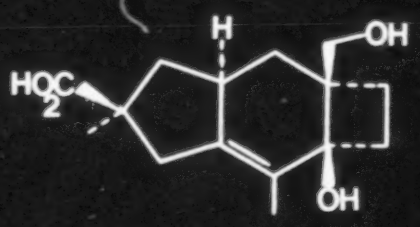

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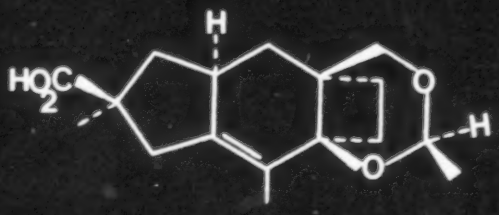

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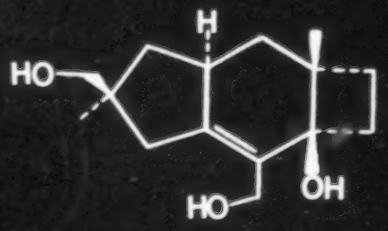

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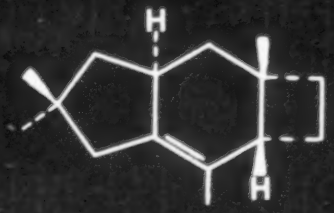

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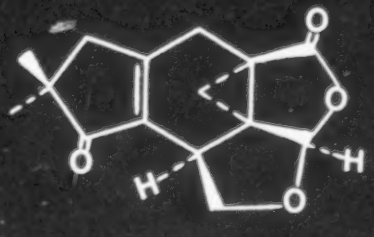

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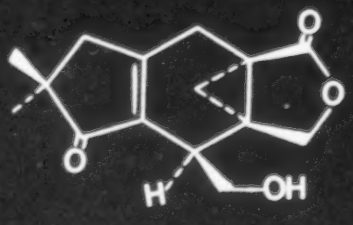

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DISCUSSION

S. purpureum was grown in malt extract-dextrose-peptone liquid culture. A study was performed to establish the optimum time required for the growth of the fungus. This revealed that a period of 30 days was the optimum time required to produce the maximum yield from the culture broth extract. Extraction with ether and ethyl acetate provided the crude metabolites. This mixture caused silvering of the foliage in mountain ash seedlings.[21] Furthermore, this extract showed good activity against Staphylococcus aureus and Candida albicans.

The crude extract of S. purpureum contained a complex mixture of many compounds as evidenced by thin layer chromatography (tlc). In an attempt to facilitate the isolation of individual components, the crude extract was separated into acidic, basic and neutral fractions. The bulk of the material was contained in the acidic and the neutral fractions. The basic fraction was negligible.

1. Acidic Compounds

a. Sterpuric acid

Column chromatography of the acidic fraction gave a small amount of a compound which after recrystallization from ethyl acetate gave a nicely crystalline compound with the melting point (mp) of 203-207° and

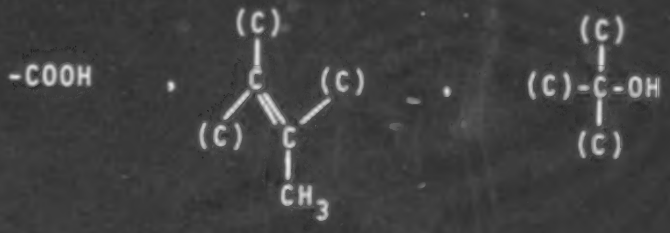
the specific rotation, $[\alpha]_D^{20}$ = +72 (CH_3OH) for which we propose the name sterpuric acid (Stereum purpureum). The molecular formula of this compound was determined by high resolution mass spectroscopy (hrms) to be $C_{15}H_{22}O_3$. The infrared (ir) spectrum shows absorptions at 3020-3600 and 1700 cm^{-1}. The 100 MHz proton magnetic resonance (^1H nmr) spectrum exhibits a one proton signal at δ 0.88 as a doublet of doublets, two quaternary methyl groups at δ 1.20 and 1.32, and a vinyl methyl at δ 1.62. The rest of the spectrum consists of a series of unresolved peaks.

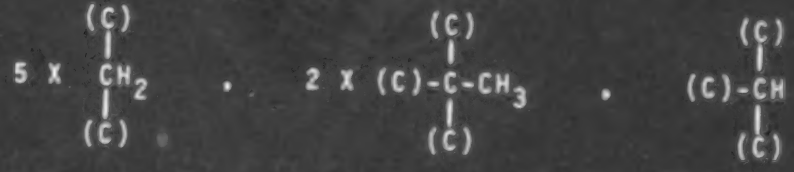
The Carbon-13 magnetic resonance (^{13}C nmr) spectrum of methyl sterpurate, the latter obtained by treatment of sterpuric acid with diazomethane shows downfield signals at δ 176.6 (s) corresponding to a carbomethoxyl carbonyl group, two low intensity signals at δ 138.3 (s) and 127.8 (s) representing the two carbon atoms of a fully substituted double bond and another singlet at δ 73.4 indicative of a tetrasubstituted carbon bearing an oxygen atom. Furthermore, the spectrum shows signals corresponding to five methylene groups, one methine carbon, two quaternary carbons and three methyl groups.

The tertiary nature of the hydroxyl group in methyl sterpurate was demonstrated chemically by its resistance to Jones' oxidation. Furthermore, acetylation of methyl

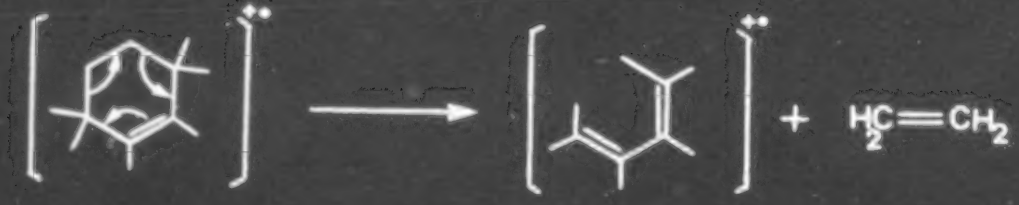
sterpurate required treatment of this compound with acetic anhydride and pyridine at room temperature for 16 days.

The spectral information and chemical properties presented thus far indicated that sterpuric acid is a tricyclic sesquiterpene with the functionality summarized below.

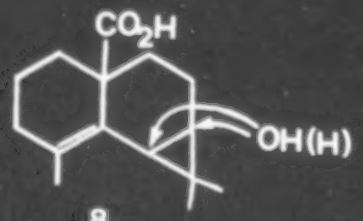




The mass spectra of sterpuric acid and its derivatives all display an intense peak at M^+-28, shown by high resolution measurements to be due to the loss of ethylene, at first suggestive of a retro Diels-Alder fragmentation. This along with the fact that the ^1H nmr spectrum of sterpuric acid has a one proton signal at δ 0.88 suggested a β-maaliene-type skeleton 8.[22] However, the assumed cyclopropane proton at δ 0.88 has two large coupling constants of 11 and 13 Hz, impossible to incorporate into this maaliene type structure.



M^+ M^+-28



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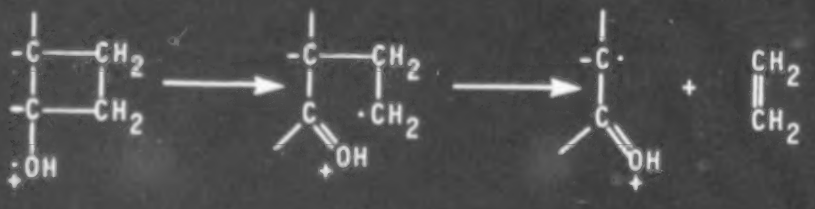
It was then found that methyl dihydrosterpurate, where the carbon-carbon double bond is reduced, also shows a base peak in the high resolution mass spectrum at M^+-28 corresponding to the loss of ethylene, ruling out the retro Diels-Alder fragmentation. This then suggested that a four-membered ring should be considered, the loss of ethylene in the ms possibly assisted by the tertiary hydroxyl group as shown.



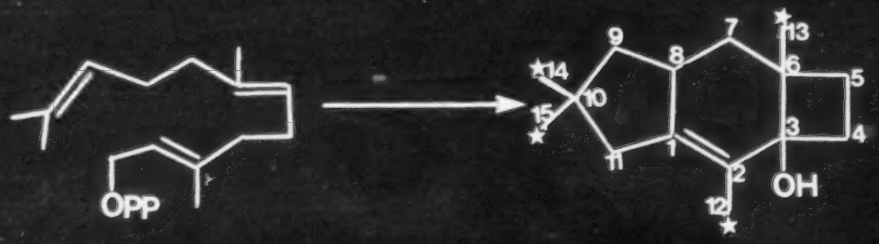
At this point it seemed apparent that sterpuric acid possesses a carbon skeleton different from that of

any of the known sesquiterpenes.

The 400 MHz ^1H nmr spectrum of sterpuric acid (Fig. 1) (which became available in early 1979) proved to be extremely informative. It not only provided better resolution for the many unresolved signals, but it also made possible the assignment of the coupling partner(s) of the vast majority of the hydrogens by double irradiation experiments. H_A (δ 2.86) must be allylic (as evidenced by upfield shift when the carbon-carbon double bond was hydrogenated) and is part of an isolated methylene group with $J_{\underline{gem}}$ = 17 Hz, the other proton of which is H_C (δ 2.24). H_B (δ 2.61) which also must be allylic is coupled to the most upfield signal H_K (δ 0.88) with J = 11 Hz and H_H (δ 1.58) with J = 6 Hz. H_K and H_H are mutually coupled ($J_{\underline{gem}}$ = 13 Hz) and not further coupled. Likewise, H_B is coupled to H_F (δ 1.86) with J = 7 Hz and H_G (δ 1.64) with J = 11 Hz and these latter two are mutually coupled ($J_{\underline{gem}}$ = 13.5 Hz) and not further coupled. The four remaining protons give rise to the signals D (δ 2.20), E (δ 1.92), I (δ 1.56) and J (δ 1.23) each of which show two large couplings and further small couplings, suggesting two contiguous methylene groups as an isolated system.

By making the assumption that sterpuric acid is derived from farnesyl pyrophosphate (9) without rearrangement, and incorporating the structural features

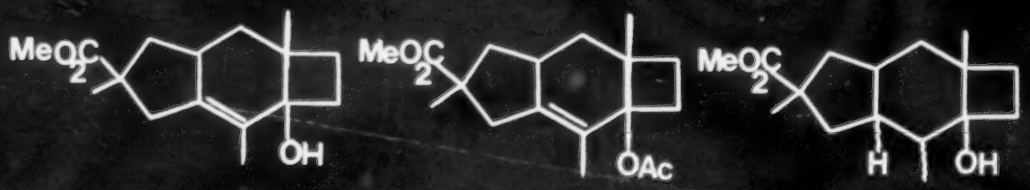
alluded to thus far, it was possible to arrive at the tentative structure 10 for sterpuric acid. At this stage it was not possible to indicate which of the starred methyl groups in 10 is a carboxyl group.



9 10

It was now necessary to prove the proposed structure by means of chemical transformations and then to determine the relative stereochemistry of the four chiral centers of sterpuric acid. Methyl sterpurate was easily prepared and routinely used for further transformations. Its ^{1}H nmr spectrum in pyridine-d_5 suggested that one of the methyl groups was on C-6 (the pyridine shift studies of methyl sterpurate will be discussed in more detail later). Acetylation of methyl sterpurate (11) under normal conditions (acetic anhydride-pyridine, room temperature) occurred very slowly to give methyl O-acetylsterpurate (12). However, the acetylation reaction could be forced to completion in three days when methyl sterpurate was treated with acetic anhydride in the presence of 4-N,N-dimethylamino-

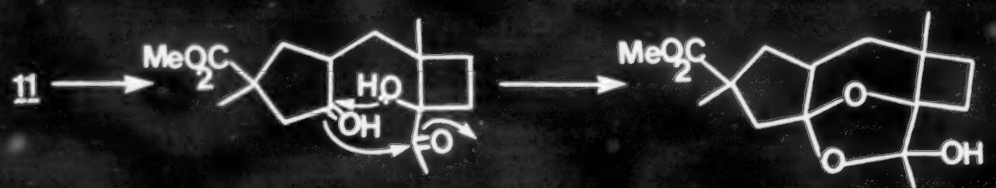
pyridine and triethyl amine in refluxing ether.[23] Hydrogenation of the carbon-carbon double bond required 60 psi pressure in the presence of excess palladium on powdered charcoal at room temperature for seven days. As indicated earlier the product of this reaction, methyl dihydrosterpurate (13), loses ethylene in the mass spectrum.



11 12 13

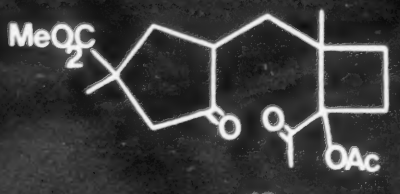
Ozonolysis[24] of methyl sterpurate (11) proceeded with the uptake of two oxygens as evidenced by high resolution ms. The ir spectrum of the product, however, displays only one carbonyl absorption at 1745 cm^{-1}. The ^1H nmr spectrum shows that the olefinic methyl group is no longer present as such, but there is also no signal representative of a methyl ketone. Instead, a methyl singlet appears at δ 1.39. It seems that the ozonolysis of 11 proceeded as expected to give the desired diketone 14, but the reaction did not stop at this stage. The presence of the tertiary hydroxyl group combined with the relative ease of formation of

five- and six-membered rings caused participation of the hydroxyl function in further reaction of 14 to form double hemiacetal 15 as indicated by the arrows in 14.



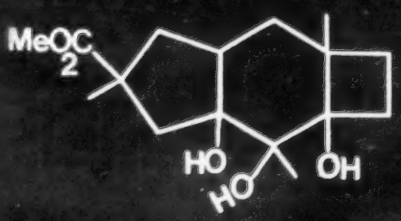
14 15

In order to circumvent this side reaction, the ozonolysis was carried out on methyl 9-acetylsterpurate (12) which contains no free hydroxyl to participate in further reaction of the diketone 11 once it is formed. The cleavage product 16 shows carbonyl absorptions at 1736 and 1710 cm^{-1} in the ir spectrum and a signal corresponding to a methyl ketone (δ 2.12) in the ^1H nmr spectrum. The absorption band at 1736 cm^{-1} must represent both the methyl ester and the cyclopentanone, but because of the overlap, does not provide a good evidence for the five-membered ring in sterpuric acid.

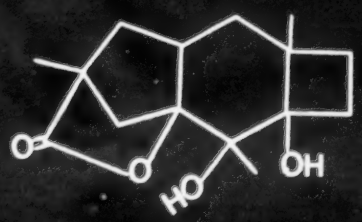


16

At this point it was decided to oxidize the carbon-carbon double bond of methyl sterpurate (11) with osmium tetroxide. It was hoped that this reaction would provide the triol 17 which then would be degraded to a cyclopentanone-cyclobutanone system. However, treatment of 11 with osmium tetroxide[25] in warm pyridine and decomposition of the osmate ester thus produced with sodium bisulfite did not result in the formation of 17 ($C_{16}H_{26}O_5$). Instead, the lactonediol 18 was produced as evidenced by spectral data. The high resolution ms of 18 indicated a molecular formula of $C_{15}H_{22}O_4$. The ir spectrum shows hydroxyl absorption at 3460 cm^{-1} and carbonyl absorption at 1775 cm^{-1} characteristic of a γ-lactone function. The ^1H nmr spectrum does not exhibit a signal corresponding to the methyl ester moiety.



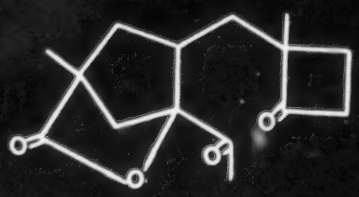
17



18

Treatment of the lactonediol 18 with paraperiodic acid[26] brought about cleavage of the vicinal diol to form a compound 19 containing cyclobutanone functionality as shown by analysis of the spectral data. The high

resolution ms of 19 indicated a molecular formula of $C_{15}H_{20}O_4$. Its ir spectrum shows carbonyl absorption bands at 1785, 1775 and 1718 cm^{-1} corresponding to cyclobutanone, γ-lactone and methyl ketone respectively. The methyl group on C-2 of methyl sterpurate, now part of a methyl ketone, appears at δ 2.35 in the ^1H nmr spectrum. The methylene group at C-4 appears as a triplet at δ 3.00 (J = 8.8 Hz), characteristic of a methylene group adjacent to the carbonyl group of a cyclobutanone.[27] The ^1H nmr spectrum also shows a broad one proton signal at δ 2.84 (proton at C-8) coupled to two otherwise isolated methylene groups (methylene groups at C-7 and C-9) consistent with the proposed structure 19. The formation of 19 provides the best direct evidence for the presence of a four-membered ring in sterpuric acid.

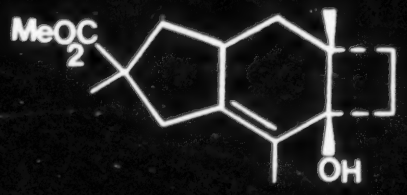


19

The results of the experiments discussed above were sufficient to characterize the skeleton and the functional groups present in sterpuric acid. At this

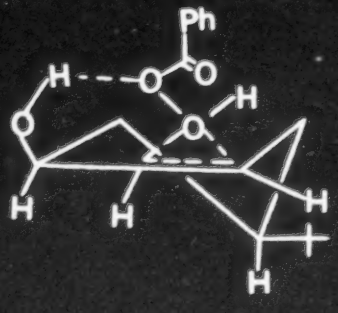
point it became necessary to establish the relative stereochemistry of the four chiral centers present in the molecule of sterpuric acid. The first piece of information concerning the stereochemical features of this compound was obtained by comparison of ^1H nmr spectra of methyl sterpurate (11) in deuterochloroform ($CDCl_3$) and pyridine-$\underline{d}_5$. It is known that pyridine is capable of specific bonding (presumably hydrogen bonding) with sites such as hydroxyl groups within a molecule. The collision complex thus formed will, in principle, have a "shape" and thus various portions of the molecule will have different spatial relationship to the complexed solvent molecules. They will therfore experience different magnetic environments due to the long range effects of the solvent molecules.[28] In the case of methyl sterpurate (11), comparison of the chemical shifts of the methyl group at C-6 in $CDCl_3$ and pyridine-$\underline{d}_5$ shows a large pyridine shift[29] from δ 1.20 in $CDCl_3$ to 1.37 in pyridine-$\underline{d}_5$ ($\delta_{CDCl_3} - \delta_{Py}$ = -0.15 ppm) indicative of <u>cis</u>-relationship between hydroxyl group at C-3 and methyl group at C-6. Likewise, the vinyl methyl exhibits a substantial pyridine shift from δ 1.67 in $CDCl_3$ to 1.85 in pyridine-$\underline{d}_5$ ($\delta_{CDCl_3} - \delta_{Py}$ = -0.18 ppm) which provides good evidence that methyl group at C-2 is in close proximity to the hydroxyl group at C-3. Thus, by the utilization of

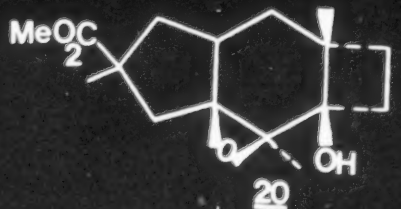
the above pyridine shift study, the structure of methyl sterpurate (11) as shown below was verified.



11

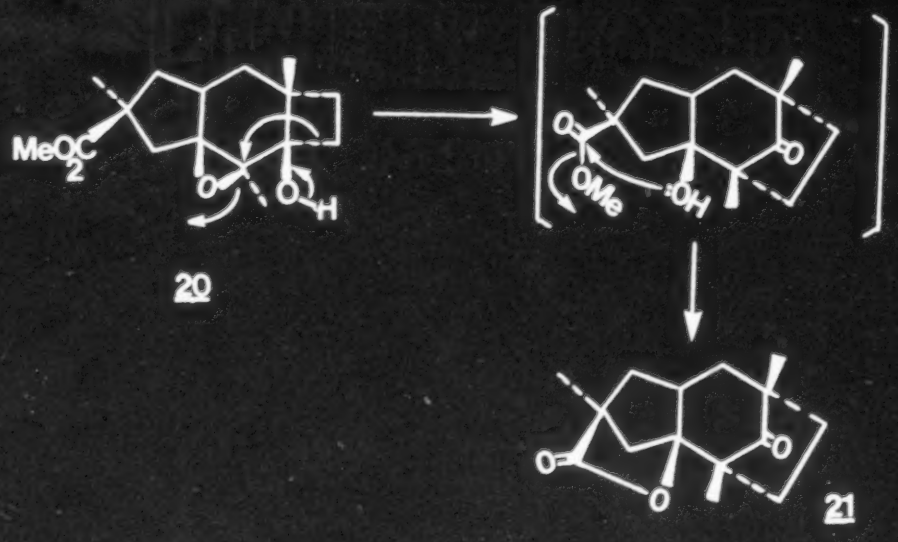
The assignment of the relative stereochemistry of the remaining chiral centers, namely, C-8 and C-10 was based on the results of the following experiments. Treatment of methyl sterpurate (11) with m-chloroperbenzoic acid in methylene chloride provided the epoxide 20. It is known[30] that in the epoxidation of allylic alcohols with peracids the addition occurs from the side of the molecule occupied by the hydroxyl group. The directive effect of the hydroxyl group has been suggested to arise because of the hydrogen bonding between the hydroxyl group and the attacking peracid as shown below. Thus, on the basis of the above argument, a cis-relationship was assigned between the C-3 hydroxyl group and the newly generated epoxide function.





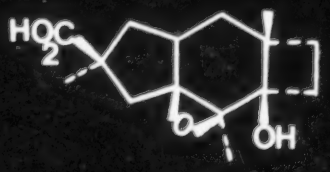
20

The establishment of the stereochemistry of the epoxide 20 provided the means for determination of the configuration of the chiral center at C-10. Treatment of 20 with p-toluenesulfonic acid in benzene at room temperature brought about smooth rearrangement of the 1-hydroxybicyclo[4.2.0]octane derivative to the crystalline bicyclo[3.2.1]octane-8-one 21, with concurrent γ-lactone formation. High resoltuion ms of 21 indicated a molecular formula of $C_{15}H_{20}O_3$. The ir spectrum shows absorption bands at 1767 (γ-lactone) and 1748 cm^{-1} (cyclopentanone) and no absorption corresponding to the presence of a hydroxyl group. The ^1H nmr spectrum exhibits three methyl singlets at δ 1.06, 1.12 and 1.33. The mechanism of formation of 21 from the epoxide 20 is shown below. This facile lactone formation indicates that the epoxide function in 20, and thus the hydroxyl group in methyl sterpurate (11), is syn to the carbomethoxyl group.



20 21

Alkaline hydrolysis of the ester 20 did not cause any participation of the carboxylate anion in epoxide ring opening, even when the reaction mixture was heated under reflux with 10% sodium hydroxide in methanol-water. This is also in agreement with the syn-relationship of the carboxyl and the epoxide (and hence the hydroxyl) functions. Treatment of the hydrolysis product 22 with p-toluenesulfonic acid in chloroform resulted in the formation of the rearrangement product 21 as expected.

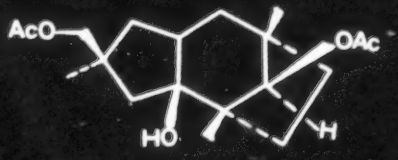


22

Evidence that the rearrangement of 20 proceeded with migration of the C-4 to C-3 bond rather than the C-6 to C-3 bond to give the bicyclo[3.3.0]octane 23 was provided by reduction of the rearranged product 21 with lithium aluminum hydride in tetrahydrofuran (THF) to the corresponding triol followed by acetylation of the latter to give the diacetate 24. The proton geminal to the secondary acetoxyl group gives rise to a sharp singlet at δ 4.72 in the ^{1}H nmr spectrum, thus ruling

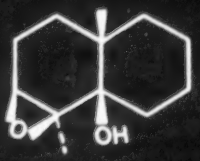
out structure 23 for the rearrangement product.

23

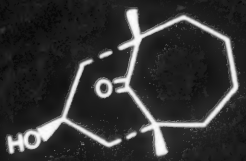


24

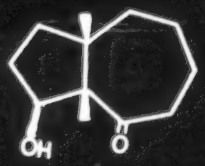
Furthermore, Marshall[31] has recently studied the pinacol-type rearrangement of 1,2-epoxy-9-decalols. Treatment of 25 with boron trifluoride etherate in methylene chloride at -20° resulted in the formation of 26 rather than 27 consistent with the results obtained in the case of 20.



25



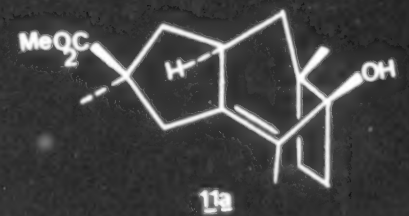
26



27

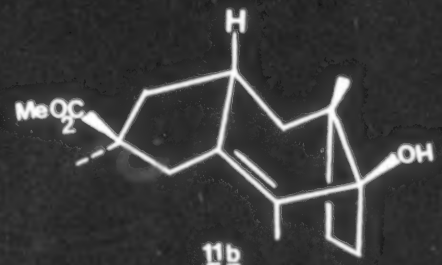
The configuration of the last chiral center at C-8 of methyl sterpurate (11) was deduced by consideration of the following observations. The two possible configurations 11a and 11b are outlined in Scheme I. The ^1H nmr spectrum of 11 shows the same chemical shift (δ 2.61) for the proton in pyridine-d_5 as in $CDCl_3$, supporting the possibility of anti relationship between

Scheme I



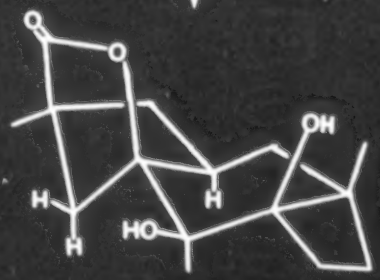
11a

vs.



11b

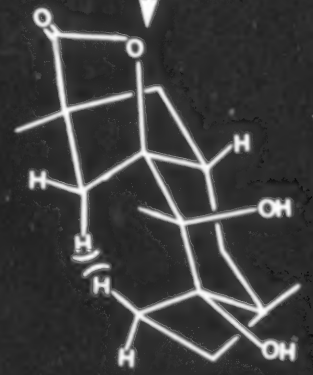
OsO_4



18a

less strained

OsO_4



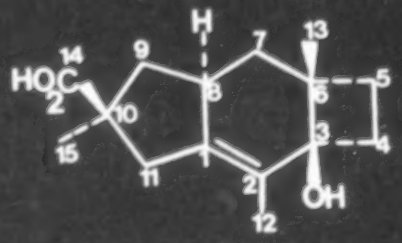
18b

the hydroxyl group at C-3 and the allylic methine proton at C-8, thus, making 11a the better candidate of the two isomers. The second piece of evidence favoring 11a over 11b was obtained from the osmium tetroxide oxidation of 11 to form the γ-lactone 18. The oxidation products from 11a and 11b would be the tetracyclic lactones 18a and 18b respectively. However, examination of models of these isomers reveals that there exists a considerable non-bonded interaction between hydrogens at C-4 and C-11 of 18b, whereas 18a is free of of such interactions.* The ease of lactone formation thus suggested that the proton at C-8 is anti to carbomethoxyl group at C-10 (and hence anti to the hydroxyl group at C-3) of methyl sterpurate (11), again making 11a the preferred isomer. Finally, confirmation of the assignment of the stereochemistry at C-8 was obtained by a pyridine shift study on hydroxydiacetate 24.

High resolution ms of 24 did not show the expected molecular formula of $C_{19}H_{30}O_5$. Instead, a peak at m/e 278 corresponding to M^+-60 was the highest mass observed. However, chemical ionization (NH_3) ms gave an M+18 peak of 356, compatible with the molecular formula of 24. The ir spectrum shows a strong absorption band at 3570 cm^{-1} for the tertiary hydroxyl group at C-1. The ^1H nmr spectrum exhibits a sharp singlet

* However, there are boat conformations of 18b which may be more stable than the chair conformation shown.

at δ 4.22 for the proton geminal to the secondary acetoxyl group, methyl singlets at δ 2.14 and 2.04 indicative of two acetate functions, and a one proton broad signal at δ 1.90 for the proton at C-8.* Since the tertiary hydroxyl group at C-1 in 24 had the same stereochemistry as the carbomethoxyl group (and hence the hydroxyl group) in methyl sterpurate (11), it was felt that the C-1 hydroxyl function could be correlated with the hydrogen at C-8 by means of a pyridine shift study. The ^{1}H nmr spectrum of 24 showed the same chemical shift (δ 1.90) for the proton at C-8 in pyridine-d_5 as in $CDCl_3$, indicating that it is not cis to the C-1 hydroxyl, and thus, it is anti to the carboxyl group in sterpuric acid. The assignment of the stereochemistry of the four chiral centers completes the assignment of structure 1 to sterpuric acid.



1

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* The configuration of the chiral center bearing the secondary acetoxyl group (C-4, sterpuric acid numbering) was assigned on the basis that hydride ion would attack from the less hindered side of 21 to afford, after acetylation, the hydroxydiacetate 24. Furthermore, the ^{1}H nmr spectrum of 24 does not show a W-coupling for the methine proton on C-4 in agreement with the proposed structure. However, the proton on C-4 undergoes a moderate pyridine shift of -0.12 ppm inconsistent with the stereochemistry assigned to this center.

It should be pointed out that structure 1 defines only the relative stereostructure of sterpuric acid and the enantiomer shown is an arbitrary choice. The relative stereochemistry can be designated as 3($\underline{R}^*$), 6($\underline{S}^*$), 8($\underline{R}^*$) and 10($\underline{S}^*$).

Structure 1 has recently been verified by an x-ray crystallographic study.[32] This study also shows that the cyclopentane ring has the envelope (Cs) conformation with C-9 serving as the flap. The cyclohexene ring has the 1,2-diplanar conformation with 0° torsional angles about C(1)-C(2) and C(2)-C(3) and the cyclobutane ring is puckered. A computer generated perspective drawing of the X-ray structure of sterpuric acid is shown in Figure 2. Hydrogens have been omitted for clarity and no absolute configuration is implied.

b. Methyl hydroxysterpurate

The isolation of the remaining acidic metabolites turned out to be very difficult. Several attempts to separate these compounds by repeated column chromatography and preparative thin layer chromatography (ptlc) using a variety of different solvent systems failed to provide a systematic method for the isolation of pure compounds with reasonable recovery of material. Therefore, in order to develop a consistent method of separation, the acidic fraction of the culture broth

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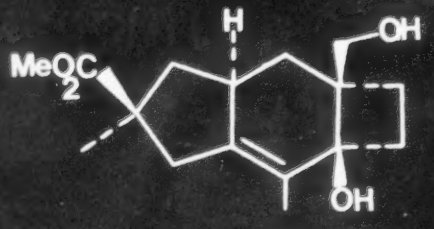
extract was treated with diazomethane to give a complex mixture of the methyl esters of the acidic compounds. Silica gel chromatography of this mixture afforded methyl sterpurate (11) as well as a more polar compound which after further purification was obtained as an oil in spectroscopically pure form.

The high resolution ms of the more polar compound showed a molecular formula of $C_{16}H_{24}O_4$. Furthermore, the mass spectrum shows a prominent peak at m/e 252 (M^+-28) corresponding to the loss of ethylene. The ir spectrum has strong hydroxyl absorption band at 3400 cm^{-1} as well as a carbonyl absorption at 1731 cm^{-1} for the methyl ester. The ^1H nmr spectrum (Fig. 3) is very similar to that of methyl sterpurate (11). However, it shows one less methyl signal and a new signal corresponding to a two proton AB quartet at δ 3.80 (J = 12 Hz), presumably due to the presence of a hydroxymethyl group. The spectrum also shows a methyl signal at δ 3.70 representing the methyl ester moiety and a one proton doublet at δ 2.86 which is part of an AB quartet with J_{gem} = 17 Hz. The other proton of this spin system appears at δ 2.28. A broad one proton signal at δ 2.63 is coupled to a one proton double doublet at δ 1.90 (J = 7 Hz) and to another double doublet at δ 1.72 (J = 12 Hz). These two latter protons are mutually coupled (J_{gem} = 13 Hz) and not further

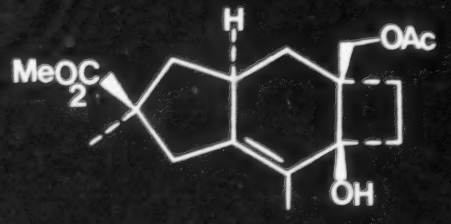
coupled. The broad signal (δ 2.63) is also coupled to another otherwise isolated methylene group, the components of which appear as a doublet of doublets at δ 1.50 (J = 7 Hz) and another doublet of doublets at δ 1.20 (J = 11 Hz) with J_{gem} = 13 Hz. The spectrum also shows a signal at δ 1.65 for a vinyl methyl group and a methyl singlet at δ 1.36. The four remaining protons give rise to signals at δ 2.23 (one proton multiplet), 2.02 (one proton multiplet), and 1.48 (a two proton complex multiplet).

The molecular formula of this new methyl ester, and the loss of ethylene in the mass spectrum suggested the sterpuric acid type of skeleton. As mentioned earlier, the ^1H nmr spectrum of this compound is quite similar to that of methyl sterpurate (11) except that the signal for the methyl group at C-6 (δ 1.20) is replaced by a signal for a two proton AB quartet at δ 3.80. Structure 28 and the name methyl hydroxysterpurate is assigned to this new compound.



28

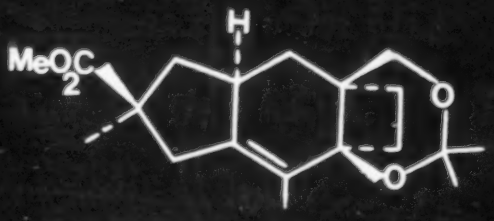
Treatment of methyl hydroxysterpurate (28) with acetic anhydride-pyridine at room temperature for a short period afforded the monoacetyl derivative 29 as indicated by spectral data. The molecular formula was $C_{18}H_{26}O_5$ by high resolution ms. The ir spectrum shows a hydroxyl absorption band (3340 cm^{-1}) indicating the presence of the tertiary hydroxyl group. The ^1H nmr spectrum exhibits a new three proton singlet at δ 2.08 for the methyl group of the acetate function and the two proton AB quartet originally at δ 3.80 in methyl hydroxysterpurate undergoes an acetate shift ($\delta_{alc.} - \delta_{acetate}$ = -0.5 ppm) to give a signal at δ 4.31.



29

The cis-relationship of the C-3 and C-6 substituents determined by study of the pyridine shift of the C-6 methyl in the case of methyl sterpurate (11) was firmly established by the following chemical transformation of methyl hydroxysterpurate (28). Brief treatment of 28 with 2,2-dimethoxypropane in the presence of p-toluenesulfonic acid brought about the forma-

tion of the acetonide 30. The high resolution ms of 30 indicated the expected molecular formula of $C_{19}H_{28}O_4$. The ir spectrum does not show hydroxyl absorption. The ^1H nmr spectrum shows two new methyl singlets at δ 1.26 and 1.39 compatible with the assigned structure 30. This acetonide formation, therefore, confirmed the 1,3-relationship of the two hydroxyl groups and the cis-nature of the ring fusion.

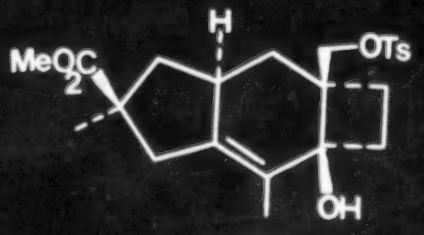


30

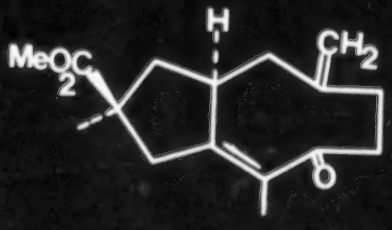
Several attempts were made to transform the hydroxymethyl group of methyl hydroxysterpurate (28) to a methyl group, thus correlating 28 with methyl sterpurate (11). These were all unsuccessful, partly because of the tendency of the hydroxymethylcyclobutanol system to undergo ring cleavage.

Tosylation of methyl hydroxysterpurate (28) was achieved by treatment of 28 with p-toluenesulfonyl chloride in the presence of sodium hydride in ether at -10° followed by room temperature workup.[33] Chromatography of the crude product provided the tosylate 31, along

with small amounts of the fragmentation product 32 (see below). The high resolution ms of 31 indicated a peak at m/e 416 corresponding to M^+-18 for $C_{23}H_{28}O_5S$. The ^1H nmr spectrum exhibits three new signals, two of which appear as doublets in the aromatic region at δ 7.79 (J = 8 Hz) and 7.33 (J = 8 Hz), the third a methyl singlet at δ 2.45, thus, confirming the presence of the tosyl group. The AB quartet bearing the primary hydroxyl group in 28 is now shifted downfield to δ 4.21 (J = 9 Hz).



31



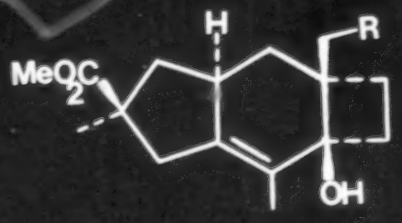
32

Reactions designed to bring about replacement of the tosyl group with hydrogen invariably led to fragmentation. Thus, treatment of 31 with sodium iodide and zinc dust in hexamethylphosphorous triamide[34] (HMPA) gave 32 as the only isolable product. A molecular formula of $C_{16}H_{22}O_3$ was obtained for 32 by high resolution ms. The ir spectrum shows absorption bands at 1730 (methyl ester) and 1650 (α,β-unsaturated ketone) and no absorption due to the presence of a hydroxyl

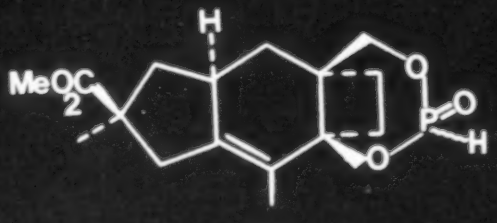
group. The ^1H nmr spectrum exhibits two downfield one proton singlets at δ 4.66 and 4.60 corresponding to the two protons of the newly generated exocyclic methylene group.

Similarly, attempts to bring about the transformation of the tosylate 31 to the bromide 33 (R = Br) by treatment of 31 with lithium bromide[35] in acetone at room temperature gave only 32. Lithium aluminum hydride reduction of 31 led to the formation of several products, the major component of which was the allylic alcohol resulting from reduction of 32. In another attempt to transform the primary hydroxyl function of methyl hydroxysterpurate (28) to the corresponding bromide 33 (R = Br), the alcohol 28 was treated with phosphorous tribromide in dry pyridine.[36] The analysis of the spectra obtained for the product of this reaction did not indicate the formation of the desired bromide 33 (R = Br). Instead, the cyclic phosphite 34 was obtained as a mixture of isomers. The high resolution ms indicated a molecular formula of $C_{16}H_{23}O_5P$. The ir spectrum shows bands at 2420 (P-H) and 1435 cm^{-1} (P = O) and shows no hydroxyl absorption. The ^1H nmr spectrum shows two downfield doublets at δ 6.88 and 6.13 both of which having a very large coupling constant (J = 96 Hz) characteristic of phosphorous-hydrogen coupling. It also shows two AB quartets at δ 5.49 (J = 9.6 Hz) and 4.46

(J = 7.2 Hz). The spectrum also shows a doubling of most of the signals including the vinyl methyl (δ 1.68 and 1.69) and the quaternary methyl group at C-10 (δ 1.35 and 1.36), suggesting the presence of two isomers in 34.



33

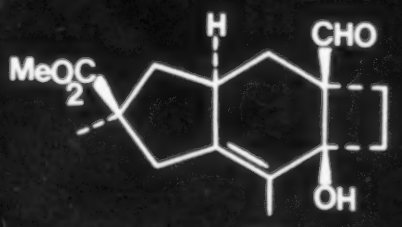


34

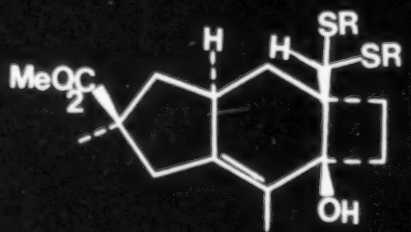
At this point, several attempts were made to bring about the replacement of the primary hydroxyl function in 28 with chlorine to afford 33 (R = Cl). Treatment of 28 with carbon tetrachloride—tri-n-octyl phosphine[37] or carbon tetrachloride—triphenyl phosphine[38] at room temperature for long periods did not produce the desired chloride 33 (R = Cl). Heating of the reaction mixtures caused the formation of complex mixtures.

Another approach for the transformation of 28 to methyl sterpurate (11) involves the oxidation of the primary alcohol function in 28 to the corresponding aldehyde 35. The resulting aldehyde might then be transformed into a thioacetal 36. Treatment of the latter with Raney nickel could provide methyl sterpurate

(11). However, treatment of 28 with pyridinium chlorochromate[39] in methylene chloride at room temperature gave a complex mixture as evidenced by tlc.



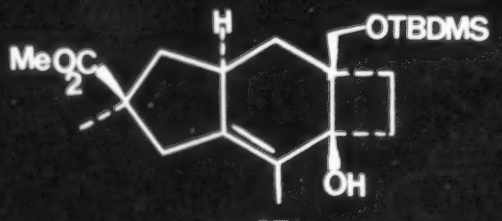
35



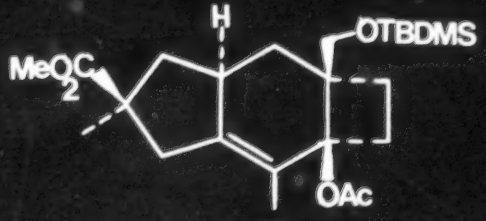
36

At this point, it seemed that the presence of the tertiary hydroxyl group at C-3 of methyl hydroxysterpurate (28) was one of the major sources of complications because of its 1,3-relationship with the primary hydroxyl group. It was evident now that the C-3 hydroxyl group facilitated the fragmentation of the tosylate 31 and also prevented the formation of the primary bromide at C-13 when 28 was treated with phosphorous tribromide. In order to circumvent this problem, it was decided to block the primary hydroxyl group with an acid sensitive protecting group and the tertiary hydroxyl group as its acetate. It was hoped that once this goal was achieved, the protecting group of the primary alcohol could selectively be removed and the resulting hydroxyl function at C-13 converted to the corresponding tosylate or bromide. Treatment

of 28 with t-butyldimethylchlorosilane[40] (TBDMSCl) in N,N-dimethylformamide (DMF) in the presence of imidazole at room temperature gave the desired t-butyldimethylsilyl ether 37. The spectral data obtained for this compound were consistent with the structure 37. Nevertheless, attempts to prepare the acetate 38 utilizing the previously described procedure[23] were unsuccessful, presumably because of the added steric hindrance caused by the bulky protecting group in 37.



37

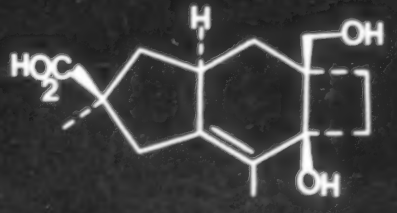


38

At this point, due to the paucity of starting material, the direct correlation of methyl hydroxysterpurate (28) with methyl sterpurate (11) was not further pursued.

As the final note, it should be pointed out that in one instance hydroxysterpuric acid (2) itself was isolated. Repeated chromatogrpahy of the crude acidic mixture and crystallization of the material thus obtained gave a very small quantity of hydroxysterpuric acid (2) as white crystals with mp 60-63°. The high

resolution ms indicated the expected molecular formula of $C_{15}H_{22}O_4$. The mass spectrum also shows an intense peak at m/e 238 (M^+-28) corresponding to the loss of ethylene. The ir spectrum shows absorptions at 3360 and 1700 cm^{-1} (carboxylic acid). The ^1H nmr spectrum of 2 is quite similar to that of methyl hydroxysterpurate (28) except that the signal for the methyl ester moiety (δ 3.70) is absent and it has three exchangeable hydrogens. The naturally occurring compound 2 was identical in all respects (ms, ir, ^1H nmr, tlc) with a sample prepared from the alkaline hydrolysis of methyl hydroxysterpurate (28).

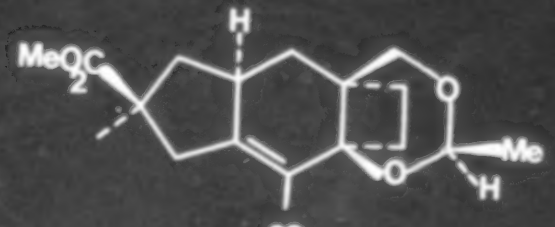


2

c. Methyl hydroxysterpurate ethylidene acetal

Fractions from chromatography of the crude acidic metabolites gave a solid material which was slightly less polar than sterpuric acid (1) and which seemed to be pure on tlc. However, treatment of this material with diazomethane gave an oil which contained several impurities (tlc). Repeated chromatography of this

mixture gave a pure colorless oil with a molecular formula of $C_{18}H_{26}O_4$ as indicated by high resolution ms. The mass spectrum also shows a prominent peak at m/e 278 (M^+-28) due to the loss of ethylene. The ir spectrum shows ester absorption (1731 cm^{-1}) and lacks a hydroxyl absorption band. The ^1H nmr spectrum (Fig. 4) of this compound is very similar to that of the isopropylidene derivative 30 (acetonide of methyl hydroxysterpurate), except that the two methyl singlets of the gem-dimethyl group of 30 are replaced by a methyl doublet at δ 1.30 J = 5 Hz) and a one proton quartet at δ 5.05 (J = 5 Hz) characteristic of methine proton geminal to two oxygens.[41] The similarity of the spectral properties of 30 and those of the newly isolated ester made it possible to assign the structure 39 to the latter, methyl hydroxysterpurate ethylidene acetal. Furthermore, the validity of the proposed structure 39 and the stereochemistry of the new chiral center were firmly established by chemical transformations.

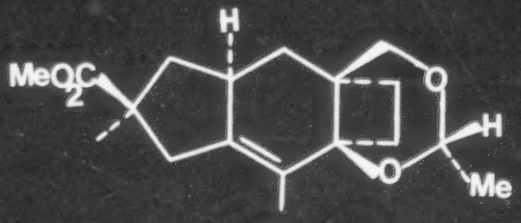


39

Treatment of 39 with 10% aqueous hydrochloric acid in THF at room temperature brought about the hydrolysis of the acetal grouping. The spectral properties of the product thus formed were identical in all respects (ms, ir, nmr) with those of methyl hydroxysterpurate (28).

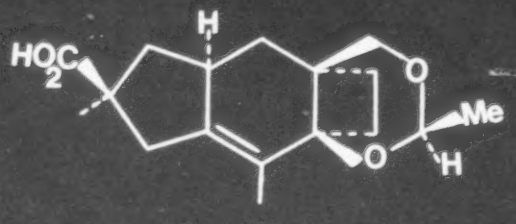
After achieving direct correlation of methyl hydroxysterpurate ethylidene acetal (39) with methyl hydroxysterpurate (28), it was necessary to determine the stereochemistry of the chiral center at the acetal functionality. Treatment of 28 with acetaldehyde in the presence of p-toluenesulfonic acid at room temperature gave a mixture of two isomeric ethylidene acetals 39 and 40 (about 1:1 ratio) along with some acetaldehyde trimer. The separation of the two epimers turned out to be very tedious because the two epimers and the acetaldehyde trimer had very similar R_f values in a variety of different solvent systems. Nevertheless, repeated ptlc and column chromatography provided small quantities of the two epimers. The less polar epimer was identical in all respects (ms, ir, ^{1}H nmr, tlc) with methyl hydroxysterpurate ethylidene acetal (39). The high resolution ms of the unnatural epimer (more polar) indicated the same molecular formula as 39. The ir spectrum is quite similar to that of 39. However, the ^{1}H nmr spectrum of the unnatural acetal shows a one proton quartet at δ 4.57 (J = 5 Hz) and

a methyl doublet at δ 1.25 (J = 5 Hz). Since the unnatural acetal shows both the methyl signal (δ 1.25) and the methine signal (δ 4.57) at higher field than those of the natural epimer (δ 1.30 and 5.05, respectively) it was possible, with the aid of ^{1}H nmr studies reported in the literature[42,43] to conclude that the methyl group in the natural acetal 39 is axial whereas in the unnatural epimer 40 it is equatorial.



40

Finally, the fact that only one of the epimers was isolated from the natural source, and both were formed during the laboratory preparation, suggests that hydroxysterpuric acid ethylidene acetal (3) is not an artifact produced during the isolation process.



3

2. Neutral Compounds

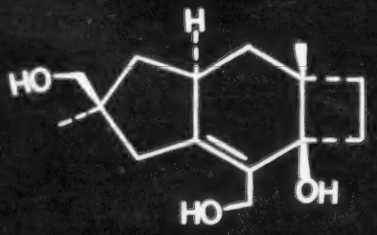
Separation of the compounds constituting the neutral fraction of S. purpureum culture broth extract proved to be very difficult due to the presence of many components. Repeated column chromatography and ptlc provided no pure compounds in sufficient quantity to be studied. The spectral data obtained for several of the assumed pure samples showed that they were in fact mixtures of compounds. Nevertheless, a fortunate incident allowed us to isolate two crystalline compounds. Occasionally, when the neutral fraction was left to stand at room temperature for a prolonged period of time, a white precipitate was formed. Separation of this material from the rest of the mixture gave a solid which consisted of mainly two components (separation of the components and then characterization will be discussed in more detail later). Furthermore, the advent of a new chromatographic technique[44] (flash chromatography) proved to be helpful in the isolation of another component of the neutral fraction.

a. Sterpurene-3,12,14-triol

The metabolite obtained by flash chromatography of the neutral fraction was further purified by column chromatography and recrystallization. The compound,

mp 146-148°, had the molecular formula $C_{15}H_{24}O_3$ as indicated by high resolution ms. The mass spectrum shows an intense peak at m/e 224 (M^+-28) due to the loss of ethylene, now a characteristic feature of the sterpurane skeleton. The ir spectrum shows a very strong absorption band at 3300 cm^{-1} corresponding to the presence of hydroxyl groups. The ^1H nmr spectrum (Fig. 5) shows AB quartets at δ 4.22 (J = 12 Hz) and 3.39 (J = 12 Hz), indicative of the presence of two isolated hydroxymethyl groups. As in the case of sterpuric acid and its congeners the ^1H nmr spectrum of this neutral compound shows a broad one proton signal at δ 2.62 corresponding to the allylic methine proton at C-8. The highest field signal at δ 0.91, a doublet of doublets (J's 11 and 13 Hz), again is suggestive of the sterpurane skeleton. The spectrum also shows a broad three proton signal at δ 2.86 which disappears on deuterium exchange experiment indicating the presence of three hydroxyl groups. Methyl singlets appear at δ 1.21 and 1.10 and there is no signal representative of a vinyl methyl moiety. On the basis of the spectral data obtained for this new metabolite and the similarity of its spectral properties with those of sterpuric acid, the structure 4 and the name sterpurene-3,12,14-triol were assigned to this compound. The chemical transformations described below are consistent

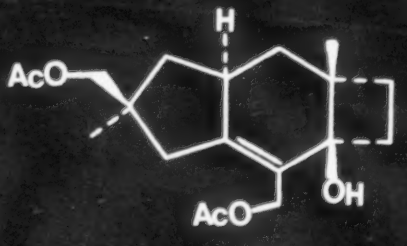
with this assignment.



4

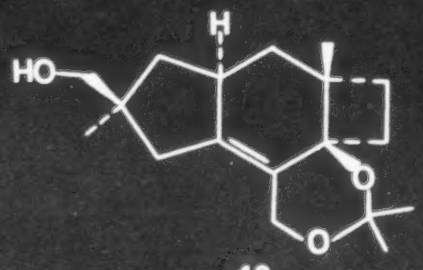
Treatment of 4 with acetic anhydride-pyridine gave an O,O-diacetyl compound 41. The highest peak in the high resolution ms corresponded to $C_{19}H_{26}O_4$ (M^+-H_2O). The ir spectrum shows acetate carbonyl absorption (1737 cm^{-1}) and a hydroxyl absorption band at 3450 cm^{-1} indicating the presence of a tertiary hydroxyl group. The ^1H nmr spectrum of 41 proved to be very informative, allowing assignment of all the signals (the spectrum will also be discussed in part II of the thesis). It exhibits methyl singlets at δ 2.08 and 2.06 for two acetate functions introduced during acetylation. The signal for the C-12 protons appears as an AB quartet at δ 4.72 ($\delta_{alc}-\delta_{acetate}$ = -0.50 ppm) and the AB quartet for protons at C-14 has a chemical shift of δ 3.90 ($\delta_{alc}-\delta_{acetate}$ =-0.51 ppm). Furthermore, the ^1H nmr spectrum of 41 in pyridine-d_5 shows a large pyridine shift ($\delta_{CDCl_3}-\delta_{Py}$ = -0.46 ppm) for the protons at C-12 and a moderate pyridine shift ($\delta_{CDCl_3}-\delta_{Py}$ = -0.2 ppm)

for the C-13 protons indicating that the hydroxymethyl group at C-2 and the methyl group at C-6 in sterpurene-3,12,14-triol(4) are proximate to the tertiary hydroxyl group at C-3.



41

The 1,3-relationship of the C-3 and C-12 hydroxyl groups of sterpurene-3,12,14-triol (4) was unambiguously established by treatment of 4 with 2,2-dimethoxypropane in the presence of p-toluenesulfonic acid to provide the acetonide 42. The molecular formula $C_{18}H_{28}O_3$ was revealed by high resolution ms. The ir spectrum has an absorption band at 3400 cm^{-1} (hydroxyl group) and the ^1H nmr spectrum shows new methyl singlets at δ 1.44 and 1.25 consistent with structure 42.



42

It should be pointed out that the assignment of the β-configuration to the hydroxymethyl group at C-10 is made on the assumption that the oxygenation pattern is the same as for sterpuric acid.

b. Lactone "A" and lactone "B"

The solid material obtained by precipitation from the neutral fractions of the broth extract mentioned earlier contained two major components as evidenced by tlc. Silica gel chromatography of this mixture provided the crystalline compounds.

Recrystallization of the less polar compound gave fine needles with mp 228-232°. The molecular formula was shown to be $C_{15}H_{16}O_4$ by high resolution ms, again suggesting the sesquiterpenoid nature of the compound. Interestingly, there is a prominent peak at m/e 232 (M^+-28) in the mass spectrum. However, this is not due to the loss of ethylene, but rather corresponds to the loss of carbon monoxide as revealed by high resolution ms. The ir spectrum shows carbonyl absorption bands at 1771, 1702 and 1650 cm^{-1} and lacks hydroxyl absorption. The absorption band at 1771 cm^{-1} is characteristic of a γ-lactone functionality, and the bands at 1702 and 1650 cm^{-1} are assigned to a 2-cyclopentenone system. The ultraviolet (uv) spectrum shows an absorption maximum at 239 nm, supporting the presence of an α,β-

unsaturated carbonyl moiety (2-cyclopentenone) in the molecule.

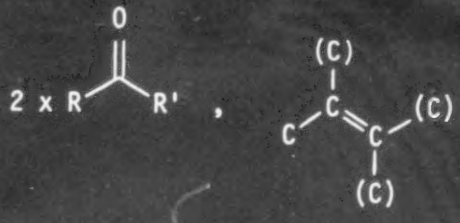
The ^1H nmr spectrum (Fig. 6) consists of a series of well resolved peaks which allowed the assignment of the coupling partner(s) of virtually all of the protons by double irradiation experiments. The spectrum shows a one proton singlet at δ 5.72 due to a methine proton geminal to two oxygen atoms. The signal at δ 3.66 is a one proton doublet of doublets coupled to two other one proton signals which appear as a doublet of doublets at δ 4.55 (J = 8 Hz) and 3.52 (J = 8 Hz). The two latter protons are mutually coupled (J_{gem} = 8 Hz) and not further coupled. The above spin system is, therefore, an ABM system consisting of the split two proton doublet of an AB quartet (centered at δ 4.03) and a doublet of doublets at δ 3.66. The ^1H nmr spectrum also shows a one proton signal at δ 3.06 which must be allylic and is a part of an isolated methylene group (J_{gem} = 20 Hz), the other proton of which appears at δ 2.84 (i.e., an AB quartet centered at δ 2.95). The two proton signal at δ 2.43 is also allylic and appears as an AB quartet (J_{gem} = 20 Hz). Two doublets which appear at δ 1.82 and 1.39 also constitute an AB quartet (centered at δ 1.60) with J_{gem} = 6 Hz. The small geminal coupling obtained for this isolated methylene group suggested the presence of a 1,1,2,2-tetrasubsti-

tuted cyclopropyl functionality. Finally, two methyl singlets appear at δ 1.14 and 1.13 in the ^{1}H nmr spectrum.

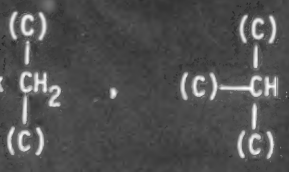
The ^{13}C nmr spectrum shows singlets at δ 210.8 (cyclopentenone carbonyl), 175.1 (γ-lactone carbonyl), 165.4 (β-carbon of a 2,3-disubstituted 2-cyclopentenone) and 132.7 (α-carbon of a 2,3-disubstituted 2-cyclopentenone), all in good agreement with values reported in the literature.[45] The spectrum also shows signals at δ 105.4 (d) corresponding to a carbon bearing two oxygen atoms, 74.9 (t) representing a methylene group bearing an oxygen atom, 46.6 (t), 44.5 (s), 40.6 (s), 31.7 (d), 28.1 (t), 25.1 (2xq), 24.5 (s) and a triplet at 19.9 corresponding to the cyclopropane methylene group.

The spectral information presented above may be summarized in terms of the parts shown below.

molecular formula: $C_{15}H_{16}O_4$, number of unsaturations = 8



, pentacyclic

2x quaternary methyl, 

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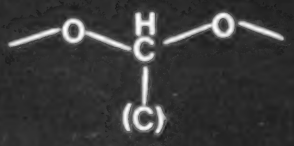
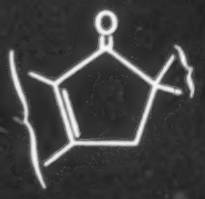


, 3x quaternary carbon

These pieces may be expanded to include the following features.



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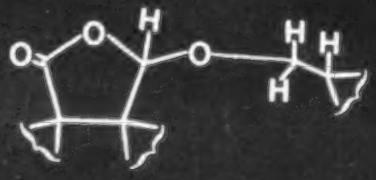
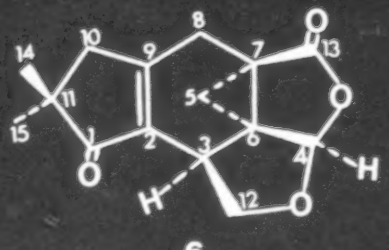
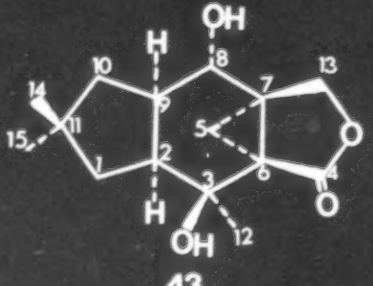


The spectral properties obtained for the above compound and their similarity with those reported for isolactarorufin[20] (43) (also a fungal metabolite) and its derivatives enabled us to assign structure 6 to this compound, known henceforth as lactone "A". It should be pointed out that 6 does not represent the absolute configuration of the molecule and the enantiomer shown is an arbitrary choice. It is, however, interesting to note that in this tight pentacyclic system, there is only one strain-free configuration possible.

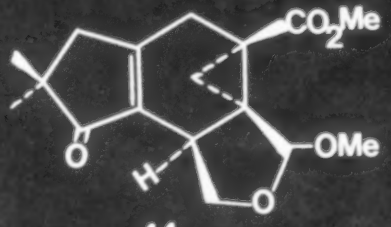


6

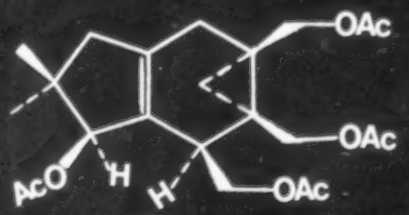


43

Hydrogenation of 6 in the presence of palladium on powdered charcoal gave a mixture of three compounds in low yield. Attempts to open the lactone ring by treatment of 6 with sodium methoxide in methanol or THF followed by addition of methyl iodide failed to provide the methyl ester 44. Treatment of 6 with aluminum hydride in THF resulted in the formation of several products. However, brief treatment of 6 with lithium aluminum hydride in THF at room temperature followed by acetylation of the crude product with acetic anhydride-pyridine gave the tetraacetate 45.*



44



45

The high resolution ms of 45 did not give the expected molecular formula of $C_{23}H_{32}O_8$, but instead it

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* The configuration of the chiral center bearing the secondary acetoxyl group was assigned on the basis that hydride ion would attack from the less hindered side of 6 to afford, after acetylation, the tetraacetate 45.

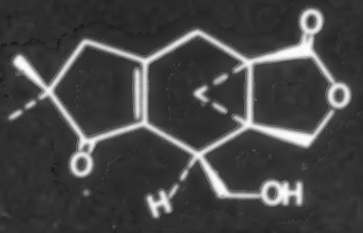
showed a peak at m/e 376 corresponding to M^+-60 due to the loss of acetic acid. The ir spectrum shows very strong carbonyl absorption at 1735 cm^{-1} and lacks a hydroxyl absorption band. The ^1H nmr spectrum exhibits a one proton singlet at δ 5.33 for the allylic proton geminal to the acetoxyl group. It also shows AB quartets at δ 2.44 (J_{gem} = 16 Hz) and 2.06 (J_{gem} = 16 Hz) corresponding to two isolated allylic methylene groups. However, the most instructive feature of this spectrum is the appearance of an AB quartet at δ 0.66 with J_{gem} = 5 Hz which clearly indicates the presence of the methylene group of a 1,1,2,2-tetrasubstituted cyclopropane moiety in lactone "A".

The more polar component from the precipitate obtained from the neutral fraction appeared as white needles with mp 210-212° after recrystallization. The molecular formula was found to be $C_{15}H_{18}O_4$. As observed in the case of lactone "A", the high resolution ms of this compound does not show a peak corresponding to the loss of ethylene. Furthermore, its ir, uv, and ^1H nmr spectra are similar in many details to those obtained for lactone "A". The ir spectrum shows absorption bands at 1760 (γ-lactone), 1695 (carbonyl group of cyclopentenone) and 1640 cm^{-1}, all observed in the case of lactone "A" at slightly higher wavenumbers. The ir spectrum also shows a strong absorption band at

3450 cm^{-1} suggesting the presence of a hydroxyl group. The uv spectrum shows an absorption maximum at 238 nm similar to that of lactone "A".

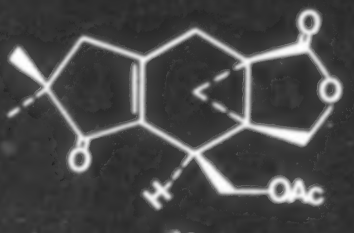
The ^1H nmr spectrum (Fig. 7) is very similar to that of lactone "A". The one proton downfield singlet at δ 5.72 in lactone "A" is no longer present, but instead there appear one proton doublets at δ 4.62 (J_{gem} = 9 Hz) and 4.33 (J = 9 Hz) representing an isolated methylene group bonded to an oxygen atom (i.e., an AB quartet centered at δ 4.47). Similar to the case of lactone "A", there is a two proton signal at δ 3.79 as a doublet of doublets (J_{gem} = 11 Hz, J_{vic} = 3.5 and 5 Hz) coupled to a broad one proton signal at δ 3.18. The allylic methylene groups appear as AB quartets at δ 2.88 (J_{gem} = 19.5 Hz) and 2.42 (J_{gem} = 18 Hz). The hydroxyl proton gives a broad signal at δ 2.06 which disappears on exchange with D_2O. The isolated methylene group of the cyclopropyl moiety exhibits doublets at δ 1.12 and 1.01 (i.e., an AB quartet at δ 1.06) with J_{gem} = 5 Hz. The methyl singlets appear at δ 1.10 and 1.09.

The spectral properties thus obtained for this compound, temporarily called lactone "B", and their similarity with those of lactone "A" made possible the assignment of structure 7 to this substance.



7

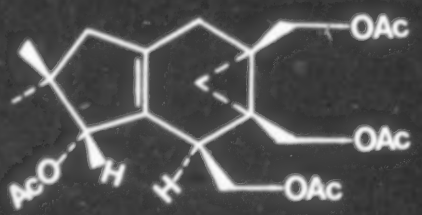
Acetylation of 7 with acetic anhydride-pyridine provided the monoacetate 46, molecular formula $C_{17}H_{20}O_5$ (high resolution ms). In the ^1H nmr spectrum of 46 the signal for the methylene group carrying the acetoxyl function appears at δ 4.27 ($\delta_{alc}-\delta_{acetate}$ = -0.38 ppm).



46

Correlation of lactone "B" with lactone "A" was obtained by treatement of lactone "B" (7) with lithium aluminum hydride and acetylation of the crude product as described for lactone "A" (6). The purified product has identical ms, ir and tlc as 45. However, the ^1H nmr spectrum shows the presence of a 1:1 ratio of epimeric products (i.e., 45 and 47) based on the fact that two signals of about equal intensity appear at δ 5.35 and 5.33 for the hydrogen at the chiral

center bearing the secondary acetoxyl group. The spectrum also shows a doubling of most of the signals, including the methyl groups of the acetate functions and the geminal methyl groups on the cyclopentene ring. The formation of two epimers can be explained by two opposing factors governing the attack of hydride on the carbonyl group of the cyclopentenone moiety. These factors are attack from the less hindered side of the molecule (as observed for lactone "A") versus complex formation between the primary hydroxyl group of 7 and the reducing agent to produce the epimeric alcohol. No attempt was made to separate the epimers due to the paucity of material.



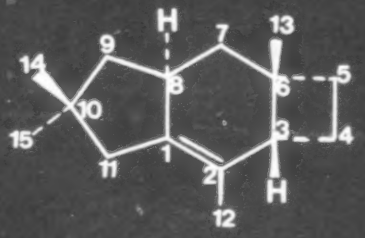
47

c. Sterpurene

The final metabolite to be discussed in this thesis was isolated from the mycelium of S. purpureum. When fresh mycelium was extracted (in a Soxhlet apparatus) and the crude extract was chromatographed on silica gel a volatile, colorless oil could be isolated. How-

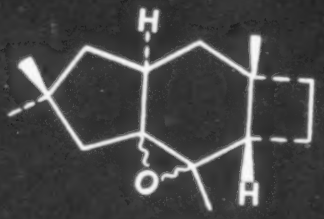
ever, this compound was not isolated in sufficient quantity to be readily studied. However, when the mycelium was kept in the freezer at -12° for three months and then extracted and purified sufficient material was obtained for examination. It was obtained as a colorless and mobile liquid. High resolution ms indicated a molecular formula of $C_{15}H_{24}$. The mass spectrum also shows a prominent peak at m/e 176 (M^+-28) corresponding to the loss of ethylene, and thus, suggesting the sterpurene type character of the compound. The ir spectrum does not show any absorption bands indicating the presence of functional groups. The ^1H nmr spectrum (Fig. 8) exhibits signals similar to those obtained for sterpuric acid (1) and its congeners discussed earlier. It contains a broad one proton peak at δ 2.65 corresponding to the proton at C-8 which is coupled to two otherwise isolated methylene groups, one of these (C-9) appears at δ 1.68 ($J_{\underline{vic}}$ = 7.5 Hz, $J_{\underline{gem}}$ = 12 Hz) and 1.58, the other (C-7) at 2.16 and 0.69 ($J_{\underline{vic}}$ = 11 Hz, $J_{\underline{gem}}$ = 13 Hz). The new allylic methine proton at C-3 appears as a doublet of doublets at δ 2.39 (J, 10.5 and 13.5 Hz). The signal for the allylic methylene group at C-11 appears at δ 2.10. The four protons on the cyclobutane ring give rise to complex multiplets at δ 1.94, 1.48, 1.44 and 0.94. The vinyl methyl group signal appears at δ 1.53. The methyl

singlet at C-6 appears at δ 1.23 and the geminal methyl groups at C-10 show singlets at δ 1.10 and 1.08. Consideration of the spectral data described above and its similarity to that of sterpuric acids and sterpurene triol suggest that this hydrocarbon has structure 5. The name sterpurene, or l-sterpurene, is suggested for this compound. The systematic name of l-sterpurene is 2,6,10,10-tetramethyl tricyclo[6.3.0.$0^{3,6}$]undec-l-ene.



5

Treatment of sterpurene (5) with m-chloroperbenzoic acid in methylene chloride gave the epoxide 48 as a single isomer. The high resolution ms indicated the expected molecular formula of $C_{15}H_{24}O$. The ir spectrum shows absorption bands at 1190 and 850 cm^{-1} indicating the presence of an oxirane ring. The ^1H nmr spectrum exhibits two one proton signals appearing as multiplets at δ 2.45 and 1.85 corresponding to the methine protons at C-8 and C-3 respectively. The spectrum also shows four methyl singlets at δ 1.19, 1.18, 1.15 and 1.12, indicating the formation of only one isomer during the epoxidation of sterpurene.



48

This compound (5) has not been further investigated, but it does appear to be the "parent" hydrocarbon of the various sterpurene metabolites.

EXPERIMENTAL

Mass spectra were recorded on an A.E.I. MS-50 mass spectrometer coupled to a DS 50 computer, or an A.E.I. MS-9 mass spectrometer (chemical ionization), and are reported as m/e (relative intensity). The formulas of all peaks reported were determined by high resolution measurements. Unless diagnostically significant only peaks at least 20% as intense as the base peak are reported. Infrared spectra were recorded on a Nicolet 7199 F.T. interferometer and ultraviolet spectra on a Unicam SP 1805 spectrometer. Optical rotations were measured on a Perkin-Elmer model 141 automatic polarimeter. Proton nuclear magnetic resonance were determined on a Varian HA-100 interfaced to a Digilab FTS/NMR-3 data system, or Bruker WH 200, or Bruker WH 400 spectrometer with TMS as internal standard. Complete spectra are reported for compound 1, 4, 5, 6, 7, 28 and 39, otherwise only diagnostically significant peaks are given. Carbon nuclear magnetic resonance spectra were measured on a Bruker HFX-90 or Bruker WH 400 spectrometer with TMS as internal standard. Melting points were recorded on a Fisher-Johns melting apparatus and are uncorrected.

Preparative tlc was carried out on 0.75 mm layers of silica gel (E. Merck, Darmstadt) containing 1% electronic phosphor (General Electric, Cleveland), and materials were detected by spraying with 30%

sulfuric acid and charring. Column chromatography was carried out using Macherey Nagel Kieselgel (0.08 mm, 100 g/g substrate). Flash chromatography was carried out using Merck silica gel 60 (0.040 - 0.063 mm). All solvents were distilled prior to use. Skellysolve B refers to Skelly Oil Company light petroleum, bp 62-70°. All compounds for which spectral data are reported showed single spots on tlc using at least two different solvent systems.

Growth of Stereum purpureum on liquid medium and isolation of the crude metabolites

The aqueous medium used for culture broth has the following composition per litre: malt extract 25 g, dextrose 13 g, peptone 0.7 g. The medium was sterilized in an autoclave at 120° for 20 minutes before use.

Slant tubes of S. purpureum (strain C-663) were obtained from Dr. Y. Hiratsuka, Northern Forest Research Center, Edmonton, and were maintained at 4° on potato dextrose agar. These were used to inoculate 500 ml Erlenmeyer flasks containing 200 ml of the above medium, and were allowed to mature for at least two weeks. To initiate large scale growths the contents of one 500 ml Erlenmeyer were blended (Waring blender) and 20 ml portions of the resulting suspension were transferred under sterile conditions to ten 2.8 ℓ

Fernback flasks containing 1 ℓ medium per flask. After 30 days growth the mycelium was removed by filtration, first through cheese cloth, then through Celite. The resulting broth was concentrated at reduced pressure to 1.5 ℓ. The concentrate was extracted with ether (3 x 1.5 ℓ) and then with ethyl acetate (2 x 1.5 ℓ). The ether extract was washed with water, then brine, dried (Na_2SO_4) and evaporated to give a viscous light brown oil (1.29 g). The ethyl acetate extract was worked up similarly to give a dark brown oil (0.3 g).

Separation of acidic and neutral compounds

The crude ether extract from above (1.25 g) was dissolved in ethyl acetate (100 ml) and the resulting solution was extracted two times with 10% sodium bicarbonate (25 ml portions). The organic phase was washed three times with water, then brine, dried (Na_2SO_4) and concentrated under reduced pressure to provide neutral materials (0.83 g) as a yellow oil. The bicarbonate extract was washed twice with chloroform, then cooled in ice bath and acidified to pH 3 with 6 M HCl. The acidified solution was extracted three times with chloroform (50 ml protions) and the combined extract was successively washed with water and brine. Drying (Na_2SO_4) and evaporation of the solvent provided acidic material (0.34 g) as a very

viscous oil.

Isolation of sterpuric acid (1)

The crude acids (0.34 g) were chromatographed over silica gel. Elution with 7.5-12.5% acetone in benzene gave crude sterpuric acid (28 mg). Repeated crystallization from ethyl acetate provided sterpuric acid (1) (18 mg) as colorless crystals, mp 203-207°.
$[\alpha]_D^{20}$ + 72° (c 0.004, CH_3OH).
IR (film): 3600-3200, 1700, 1455, 1433, 1375, 1320, 1268, 1240, 1220, 1192, 1140, 1115, 1080 cm^{-1}.
^1H NMR (CD_3OD): δ 2.86 (1 H on C-11, d, J_{gem} = 17 Hz), 2.61 (1 H on C-8, br. mult.), 2.25 (1 H on C-11, d, J_{gem} = 17 Hz), 2.20 (1 H on C-4, complex mult.), 1.92 (1 H on C-4, complex mult.), 1.86 (1 H on C-9, dd, $J_{8,9}$ = 7 Hz, J_{gem} = 12 Hz), 1.64 (1 H on C-9, dd, $J_{8,9}$ = 11 Hz, J_{gem} = 12 Hz), 1.62 (3 H on C-12, s), 1.58 (1 H on C-7, dd, $J_{7,8}$ = 6 Hz, J_{gem} = 13 Hz), 1.56 (1 H on C-5, complex mult.), 1.32 (3 H on C-15, s), 1.23 (1 H on C-5, complex mult.), 1.20 (3 H on C-13, s), 0.88 (1 H on C-7, dd, $J_{7,8}$ = 11 Hz, J_{gem} = 13 Hz). The assignments of coupling partners were confirmed by double irradiation experiments.
MS (probe, 150°): m/e Calcd. for $C_{15}H_{22}O_3$: 250.1569 found: 250.1573 (30), 222 (100), 176 (17), 175 (49), 161 (49), 136 (53), 135 (41), 91 (25).

Methyl sterpurate (11)

Diazomethane in ether (3 ml, 0.3 M) was added to a solution of sterpuric acid (25 mg) in ether-methanol (3 ml, 1:1). After 15 min. the solvents were removed under reduced pressure to provide methyl sterpurate (26 mg) as an oil which was purified by chromatography (eluent benzene-ether (19:1)) to give pure methyl sterpurate (25 mg) as an oil.

IR (film): 3460, 1731, 1455, 1433, 1376, 1318, 1268, 1220, 1192, 1142, 1115, 1078 cm^{-1}.

^1H NMR ($CDCl_3$): δ 3.70 (3 H, OCH_3, s), 2.83 (1 H on C-11, d, J_{gem} = 17 Hz), 2.54 (1 H on C-8, br. mult.), 2.23 (1 H on C-11, d, J_{gem} = 17 Hz), 1.67 (3 H on C-12, s), 1.35 (3 H on C-15, s), 1.22 (3 H on C-13, s), 0.87 (1 H on C-7, dd, $J_{7,8}$ = 11 Hz), J_{gem} = 13 Hz).

^1H NMR (Py-d_5): δ 3.66 (3 H, s), 2.98 (1 H, d, J = 17 Hz), 2.54 (1 H, br. mult.), 1.85 (3 H on C-12, s), 1.37 (3 H on C-13, s), 1.35 (3 H, s), 0.80 (1 H, dd, $J_{7,8}$ = 11 Hz, J_{gem} = 13 Hz).

MS (probe, 200°): m/e Calcd. for $C_{16}H_{24}O_3$: 264.1726 found: 264.1720 (43), 236 (100), 205 (47), 177 (25), 176 (66), 175 (64), 161 (57), 149 (28), 136 (48), 133 (26), 121 (29), 107 (29), 105 (24), 101 (34), 91 (35), 77 (22).

Methyl O-acetylsterpurate (12)

To a solution of methyl sterpurate (11, 11 mg) in ether (4 ml) was added acetic anhydride (0.15 ml), triethylamine (0.2 ml) and 4-N,N-dimethylaminopyridine (5 mg). The solution was heated under reflux for three days. Ether (50 ml) was added and the resultant solution was washed successively with 0.5 M HCl, water, and brine. Drying (Na_2SO_4), evaporation of the solvents and chromatography (eluent benzene-acetone (19:1)) gave methyl O-acetylsterpurate (12, 11 mg) as a colorless oil.

IR (film): 1737, 1460, 1430, 1370, 1240, 1130, 1090 cm^{-1}.

^1H NMR ($CDCl_3$): δ 3.68 (3 H, s), 2.90 (1 H, d, J_{gem} = 17 Hz), 2.60 (1 H, br. mult.), 2.00 (3 H, acetate methyl, s), 1.50 (3 H, s), 1.36 (3 H, s), 1.20 (3 H, s).

MS (probe, 150°): m/e Calcd. for $C_{18}H_{26}O_4$: 306.1813. found: 306.1827 (16), 278 (37), 264 (100), 246 (32), 236 (91), 205 (22), 187 (46), 186 (25), 176 (25), 175 (44), 161 (26), 159 (20), 149 (20), 135 (28), 105 (25), 91 (36), 85 (28), 79 (20), 77 (20).

Methyl dihydrosterpurate (13)

Methyl sterpurate (13 mg) in methanol (5 ml) was shaken under hydrogen (4 kg/cm^2) for 7 days in the presence of 5% Pd-C (80 mg). The suspension was then

filtered through Celite and the filtrate was evaporated under reduced pressure. Chromatography of the crude product (eluent benzene-ether (19:1)) provided methyl dihydrosterpurate (13, 5 mg) as a colorless oil.

IR (film): 3510, 1730, 1455, 1433, 1370, 1231, 1135 cm^{-1}.

^1H NMR ($CDCl_3$): δ 2.69 (3 H, s), 1.31 (3 H, s), 1.24 (3 H, s), 1.14 (3 H, d, J = 6 Hz).

MS (probe, 150°): m/e Calcd. for $C_{16}H_{26}O_3$: 266.1882. found: 266.1888 (13), 238 (100), 189 (28), 178 (22), 137 (21), 135 (60), 123 (33), 109 (33), 95 (35), 81 (29), 79 (23), 55 (38).

Ozonolysis of methyl sterpurate (11)

A solution of methyl sterpurate (6 mg) in methanol (1 ml) was cooled to -60° and ozone was bubbled in until the solution remained blue. After flushing with nitrogen at -60°, dimethyl sulfide (0.2 ml) was added and the reaction mixture was successively stirred at -10°, 0°, and room temperature for 1 h periods. The solvent was then evaporated under reduced pressure and ether (50 ml) added. The solution was washed with water and brine, then dried (Na_2SO_4). Evaporation of the solvent left an oil which was purified by chromatography (eluent benzene-acetone (19:1)) to give compound 15 as an oil (6 mg).

IR (film): 3485, 1745, 1450, 1375, 1245, 1155, 1115 cm^{-1}.

^{1}H NMR ($CDCl_3$): δ 3.74 (3 H, $OC\underline{H}_3$, s), 1.39 (3 H, methyl group of the double hemiacetal function, s), 1.16 (3 H, s), 0.88 (3 H, s).

MS (probe, 150°): m/e Calcd. for $C_{16}H_{24}O_5$: 296.1623. found: 296.1626 (2), 128 (23), 100 (92), 97 (100).

Ozonolysis of methyl 0-acetylsterpurate (12)

Methyl 0-acetylsterpurate was subjected to ozonolysis as described above for methyl sterpurate. The crude product was purified by chromatography (eluent benzene-acetone (19:1)) to provide compound 16 as a colorless solid (3 mg).

IR (film): 1736, 1710, 1455, 1440, 1375, 1245, 1170 cm^{-1}.

^{1}H NMR ($CDCl_3$): δ 3.71 (3 H, s), 2.12 (3 H on methyl ketone, s), 2.06 (3 H, s), 1.40 (3 H, s), 1.27 (3 H, s).

MS (probe, 150°): the highest peak corresponded to $M^+ - C_2H_2O$.

m/e Calcd. for $C_{16}H_{24}O_5$ ($M^+ - C_2H_2O$): 296.1624.

found: 296.1626 (100), 253 (46), 235 (31), 193 (27), 151 (21), 127 (22), 97 (48).

Osmium tetroxide oxidation of methyl sterpurate (11)

A solution of methyl sterpurate (20 mg) and osmium tetroxide (30 mg) in dry pyridine (2 ml) was stirred at 50° for 12 h, then cooled to room temperature and a

solution of sodium bisulfite (0.18 g) in water (3 ml) and pyridine (2 ml) added. This was stirred at room temperature for 3 h, then diluted with water (5 ml) and extracted three times with ether (30 ml portions). The combined ether extract was washed with water and brine, dried (Na_2SO_4) and evaporated under reduced pressure. Chromatography of the product (eluent benzene-ether (7:3)) provided lactonediol 18 (7 mg) as a colorless solid, mp 145-150°.

IR (film): 3460, 1775, 1731, 1450, 1380, 1331, 1260, 1090 cm^{-1}.

^1H NMR ($CDCl_3$): δ 2.40 (1 H on C-8, br. mult.), 2.23 (2 H, complex mult.), 2.07 (2 H, complex mult.), 1.97 (1 H, d, J = 10.5 Hz), 1.61 (1 H, dd, J's 6.5 and 14 Hz), 1.50 (1 H, mult.), 1.48 (3 H, s), 1.33 (3 H, s), 1.26 (3 H, s), 1.10 (1 H on C-7, dd, $J_{7,8}$ = 12 Hz, J_{gem} = 13 Hz).

MS (probe, 150°): m/e Calcd. for $C_{15}H_{22}O_4$: 266.1518. found: 266.1510 (1), 248 (56), 238 (87), 195 (74), 193 (24), 177 (21), 169 (30), 151 (29), 149 (29), 142 (22), 123 (100), 107 (21), 93 (22), 81 (30), 69 (29).

Periodate cleavage of diol 18

Paraperiodic acid (H_5IO_6, 20 mg) was added to a solution of 18 in dry THF (0.5 ml) and the solution was stirred at room temperature for 10 min. Ether (40 ml)

was added and the solution was washed with water and brine, then dried (Na_2SO_4) and the solvents were evaporated under reduced pressure. Chromatography of the crude product (eluent benzene-ether (9:1)) gave diketone 19 as an oil (6 mg).

IR (film): 1785, 1775, 1718, 1450, 1361, 1260, 1232, 1200, 1161 cm^{-1}.

^1H NMR ($CDCl_3$): δ 3.00 (2 H on C-4, t, J = 8.8 Hz). 2.84 (1 H on C-8, br. mult.), 2.35 (3 H on methyl ketone, s), 2.17 (1 H on C-9, dd, $J_{8,9}$ = 10.5 Hz, J_{gem} = 13 Hz), 2.01 (2 H on C-11, AB quartet, J = 10.4 Hz), 1.85 (2 H on C-5, m, $J_{4,5}$ = 8.8 Hz, J_{gem} = 11 Hz), 1.64 (2 H on C-7, d of an AB quartet, $J_{7,8}$ = 5 Hz, J_{gem} = 14.5 Hz), 1.36 (1 H on C-9, dd, $J_{8,9}$ = 4.8 Hz, J_{gem} = 13 Hz), 1.34 (3 H, s), 1.17 (3 H, s). The assignments of coupling partners were verified by double irradiation experiments.

MS (probe, 150°): m/e Calcd. for $C_{15}H_{20}O_4$: 264.1361. found: 264.1372 (15), 236 (24), 193 (20), 179 (31), 151 (26), 140 (25), 136 (74), 135 (25), 123 (23), 122 (53), 112 (23), 109 (23), 107 (32), 97 (28), 95 (26), 93 (56), 82 (55), 81 (40), 79 (24), 69 (100), 67 (31), 55 (55), 53 (23).

Epoxidation of methyl sterpurate (11)

m-Chloroperbenzoic acid (25 mg of 85%) was added to

an ice cold solution of methyl sterpurate (11, 17 mg) in methylene chloride (0.75 ml). The reaction mixture was kept at room temperature for 1 h, then diluted with ether (50 ml). This was washed successively with satd. solution of sodium bicarbonate, water, and brine. Drying (Na_2SO_4) and evaporation of the solvents under reduced pressure left an oil (17 mg) which was purified by chromatography (eluent benzene-ether (9:1)) to provide epoxide 20 as a colorless oil (15 mg).

IR (film): 3460, 1735, 1455, 1435, 1285, 1245, 1235, 1151 cm^{-1}.

^1H NMR ($CDCl_3$): δ 3.68 (3 H, s), 1.38 (3 H, s), 1.33 (3 H, s), 1.07 (3 H, s).

MS (probe, 100°): m/e Calcd. for $C_{16}H_{24}O_4$: 280.1675. found: 280.1672 (7), 252 (23), 203 (35), 183 (66), 177 (21), 175 (21), 159 (26), 151 (31), 135 (45), 125 (92), 123 (100), 111 (22), 107 (20), 93 (23), 91 (26), 77 (25), 55 (20).

Rearrangement of epoxide 20

To a solution of epoxide 20 (8 mg) in benzene (0.5 ml) was added p-toluenesulfonic acid (5 mg). The reaction was stirred at room temperature for 4 h, then methylene chloride (30 ml) was added and the resultant solution was washed successively with satd. sodium bicarbonate, water (three times), and brine. Drying

(Na_2SO_4) and evaporation of the solvents followed by chromatography (eluent benzene-ether (9:1)) gave the rearranged product 21 (5 mg) as a colorless solid, mp 142-148°.

IR (film): 1767, 1748, 1455, 1445, 1370, 1330, 1180, 1150, 1010 cm^{-1}.

^1H NMR ($CDCl_3$): δ 2.78 (1 H on C-8, m), 2.02 (2 H on C-11, AB quartet, J = 10.5 Hz), 1.37 (1 H, m), 1.33 (3 H, s), 1.12 (3 H, s), 1.06 (3 H, s).

MS (probe, 150°): m/e Calcd. for $C_{15}H_{20}O_3$: 248.1412. found: 248.1409 (26), 121 (22), 111 (100), 69 (23).

Hydrolysis of epoxide 20 and rearrangement of acid 22

A mixture of epoxide 20 (32 mg) and 10% sodium hydroxide in methanol-water (4:1, 5 ml) was heated under reflux for 3 h. Most of the methanol was removed under reduced pressure and water (10 ml) added. The aqueous solution was washed with ether, then it was cooled in ice bath and acidified with 3 M HCl. This was extracted four times with chloroform (25 ml portions) and the combined extract was washed with water and brine. Drying (Na_2SO_4) and evaporation of the solvent gave acid 22 (25 mg). A small portion (1 mg) of the acid was esterified with diazomethane to give ester 20. The remainder (24 mg) was dissolved in chloroform (1 ml) and p-toluenesulfonic acid (5 mg) added. After stirring at room

temperature for two days the reaction mixture was worked up (as described in the case of rearrangement of epoxide 20) to provide the rearrangement product 21 (6 mg), identical (tlc, IR, ^{1}H NMR, MS) with that prepared directly from epoxide 20.

Preparation of hydroxydiacetate 24

The rearrangement product 21 (4.5 mg) was dissolved in THF (1 ml) and lithium aluminum hydride (15 mg) added. After stirring at room temperature for 20 min. excess hydride was destroyed by the addition of wet ether. Ethyl acetate (40 ml) was added and the resultant solution was washed with water (three times), then with brine. Drying (Na_2SO_4) and evaporation of the solvents provided an oil (3.5 mg) which was dissolved in pyridine (0.5 ml) and acetic anhydride (0.25 ml) added. After 48 h stirring at room temperature ether (40 ml) was added and the solution was washed successively with 0.5 M HCl, water, and brine. Drying (Na_2SO_4) and evaporation of the solvents followed by chromatography (eluent benzene-ether (4:1)) gave 24 as a colorless oil (3 mg).

IR (film): 3570, 1739, 1450, 1370, 1225, 1210, 1020 cm^{-1}.

^{1}H NMR ($CDCl_3$): δ 4.72 (1 H, C<u>H</u>OAc, s), 4.00 (2 H, C<u>H</u>$_2$OAc, AB quartet, J = 10.5 Hz), 2.14 (3 H, s), 2.04 (3 H, s), 1.90 (1 H on C-8, m), 1.07 (3 H, s), 1.05 (3 H,

s), 0.92 (3 H, s).

^{1}H NMR (Py-$\underline{d}_5$): δ 4.84 (1 H, C$\underline{H}$OAc, s), 4.32 (2 H, AB quartet, J = 10.5 Hz), 2.05 (3 H, s), 2.01 (3 H, s), 1.90 (1 H on C-8, m), 1.17 (3 H, s), 1.14 (3 H, s), 0.92 (3 H, s).

Chemical Ionization (NH_3) MS shows M+18 (m/e 356) peak.

MS (probe, 150°): the highest peak corresponded to $M^+-C_2H_4O_2$; m/e Calcd. for $C_{17}H_{26}O_3$ ($M^+-C_2H_4O_2$): 278.1881 found: 278.1881 (4), 95 (100).

Isolation of methyl hydroxysterpurate (28)

Crude acidic metabolites (0.7 g) were dissolved in ether-methanol (2:1, 30 ml) and treated with excess 0.3 M ethereal diazomethane. After standing at room temperature for 15 min. the solvents were evaporated and the residue was chromatographed (eluent benzene-ether (7:3)) to give crude methyl hydroxysterpurate (0.125 g). Further purification by ptlc (triple elution with ether) and column chromatography (eluent chloroform-methanol (99:1)) provided pure methyl hydroxysterpurate (46 mg) as a colorless oil.

IR (film): 3400, 1731, 1450, 1433, 1379, 1319, 1265, 1230, 1130, 1072, 1030 cm^{-1}.

^{1}H NMR ($CDCl_3$): δ 3.80 (2 H on C-13, AB quartet, J = 12 Hz), 3.70 (3 H, OC$\underline{H}_3$, s), 2.86 (1 H on C-11, d, $J_{\underline{gem}}$ = 17 Hz), 2.63 (1 H on C-8, br. mult.), 2.28 (1 H

on C-11, d, J_{gem} = 17 Hz), 2.23 (1 H on C-4, m), 2.02 (1 H on C-4, complex mult.), 1.90 (1 H on C-9, dd, $J_{8,9}$ = 7 Hz, J_{gem} = 13 Hz), 1.72 (1 H on C-9, dd, $J_{8,9}$ = 12 Hz, J_{gem} = 13 Hz), 1.65 (3 H on C-12, s), 1.51 (1 H on C-7, dd, $J_{7,8}$ = 7 Hz, J_{gem} = 13 Hz), 1.48 (2 H on C-5, complex mult.), 1.36 (3 H on C-15, s), 1.20 (1 H on C-7, dd, $J_{7,8}$ = 11 Hz, J_{gem} = 13 Hz). The assignments of coupling partners were verified by double irradiation experiments.

MS (probe, 150°): m/e Calcd. for $C_{16}H_{24}O_4$: 280.1675. found: 280.1673 (21), 252 (52), 250 (27), 189 (96), 175 (47), 174 (100), 161 (28), 91 (21).

Acetylation of methyl hydroxysterpurate (28)

Acetic anhydride (0.25 ml) was added to a solution of methyl hydroxysterpurate (28, 15 mg) in pyridine (0.5 ml) and the solution was stirred at room temperature for 1.5 h. After removal of the solvents the crude product was chromatographed (eluent benzene-ether (9:1)) to give the monoacetate 29 as a colorless oil (13 mg).

IR (film): 3460, 1735, 1450, 1432, 1380, 1245, 1135, 1072, 1030 cm^{-1}.

^1H NMR ($CDCl_3$): δ 4.31 (2 H on C-13, s), 3.68 (3 H, s), 2.86 (1 H on C-11, d, J_{gem} = 17 Hz) 2.58 (1 H on C-8, br. mult.), 2.24 (1 H on C-11, d, J_{gem} = 17 Hz), 2.08 (3 H, acetate methyl, s), 1.65 (3 H on C-12, s), 1.34

(3 H on C-15, s), 1.01 (1 H, dd, $J_{7,8}$ = 11 Hz, J_{gem} = 13 Hz).

MS (probe, 150°): m/e Calcd. for $C_{18}H_{26}O_5$: 322.1780. found: 322.1791 (35), 294 (11), 262 (24), 234 (25), 203 (36), 202 (21), 189 (55), 175 (78), 174 (100), 159 (26), 145 (20), 91 (21).

Methyl hydroxysterpurate acetonide (30)

Methyl hydroxysterpurate (28, 15 mg) was dissolved in 2,2-dimethoxypropane (2 ml) and a catalytic amount of p-toluenesulfonic acid added. The reaction mixture was stirred at room temperature for 1 h. Ether (40 ml) was added and the resultant solution was successively washed with satd. sodium bicarbonate, water (three times), and brine. After drying (Na_2SO_4) and evaporation of the solvents under reduced pressure the crude product was chromatographed (eluent benzene-ether (9:1)) to provide the acetonide 30 as an oil (15 mg).

IR (film): 1731, 1450, 1435, 1320, 1300, 1235, 1130, 1085 cm^{-1}.

^1H NMR ($CDCl_3$): δ 3.70 (2 H, AB quartet, J = 11 Hz), 3.68 (3 H, s), 2.82 (1 H on C-11, d, J_{gem} = 17 Hz), 2.60 (1 H on C-8, br. mult.), 2.23 (1 H on C-11, J_{gem} = 17 Hz), 1.59 (3 H on C-12, s), 1.39 (3 H, acetal methyl, s), 1.36 (3 H on C-15, s), 1.26 (3 H, acetal methyl, s).

MS (probe, 100°): m/e Calcd. for $C_{19}H_{28}O_4$: 320.1988.

found: 320.1994 (6), 252 (26), 189 (56), 175 (38), 174 (100).

Methyl hydroxysterpurate p-toluenesulfonate (31)

Sodium hydride (12 mg) was added to a solution of methyl hydroxysterpurate (30 mg) in ether (2 ml) and the mixture stirred at room temperature for 12 h. The solution was then cooled to -10° and p-toluenesulfonyl chloride (22 mg) in ether (1 ml) added. Stirring was continued at -10° for 1 h, then at room temperature for an additional 1 h. Brine (5 ml) was added and the mixture was extracted three times with ether (20 ml portions). The combined ether extract was washed with water and brine, dried (Na_2SO_4) and evaporated to give the crude product which was purified by chromatography (eluent benzene-ether (19:1)) to provide the tosylate 31 as an oil (20 mg).

^{1}H NMR ($CDCl_3$): δ 7.79 (2 H, d, J = 8 Hz), 7.33 (2 H, d, J = 7 Hz), 4.21 (2 H, AB quartet, J = 9 Hz), 3.70 (3 H, s), 2.83 (1 H, d, J = 17 Hz), 2.57 (1 H, br. mult.), 2.45 (3 H, Ph-C$\underline{H}_3$, s), 1.60 (3 H, s), 1.33 (3 H, s), 0.91 (1 H, dd, $J_{7,8}$ = 11 Hz, J_{gem} = 13 Hz).

MS (probe, 200°): the highest peak corresponded to M^+-H_2O. m/e Calcd. for $C_{23}H_{29}O_5{}^{32}S$ (M^+-H_2O): 416.1657. found: 416.1659 (7), 357 (20), 244 (30), 216 (35), 203 (30), 185 (100), 184 (36), 172 (23), 157 (52), 156 (29),.

91 (35).

Earlier fractions from the chromatography gave the cleavage product 32 (1 mg) described below.

Attempted transformation of 31 to methyl sterpurate (11)

The p-toluenesulfonate 31 (7 mg) was dissolved in HMPA (2 ml) and sodium iodide (19 mg) and zinc dust (17 mg) were added. The mixture was heated at 105° for 3 h, then cooled to room temperature, and diluted with ether (40 ml). The solution was washed with water and brine, dried (Na_2SO_4) and the solvents were removed under reduced pressure. Chromatography of the crude product (eluent benzene-ether (19:1)) gave the fragmentation product 32 as a colorless oil (3 mg).

IR (film): 1730, 1650, 1450, 1373, 1240, 1105, 900 cm^{-1}.

UV (CH_3OH): λ_{max} 244 nm.

^1H NMR ($CDCl_3$): 4.66 (1 H on exocyclic methylene, d, J = 2 Hz), 4.60 (1 H on exocyclic methylene, d, J = 2 Hz), 3.72 (3 H, s), 3.10 (2 H, complex mult.), 1.82 (3 H on C-12, s), 1.26 (3 H, s).

MS (probe, 150°): m/e Calcd. for $C_{16}H_{22}O_3$: 262.1569. found: 262.1572 (50), 203 (100), 202 (44), 159 (41), 145 (42), 119 (24), 107 (23), 105 (23), 91 (23).

Attempted bromide formation with methyl hydroxysterpurate (28)

Phosphorous tribromide (50 mg) was added to an ice cold solution of methyl hydroxysterpurate (7 mg) in pyridine (0.75 ml). The reaction mixture was allowed to warm up to room temperature and stirred for 45 min. Ether (40 ml) was added and the resultant solution was washed three times with water, then with brine, and dried (Na_2SO_4). Evaporation of the solvents and chromatography of the crude product (eluent benzene-ether (9:1)) provided the phosphite 34 as an oil (4 mg).

IR (film): 2400, 1731, 1450, 1432, 1380, 1280, 1128, 1020 cm^{-1}.

^1H NMR ($CDCl_3$): δ 6.88 (1 H, PH, d, J = 96 Hz), 6.13 (1 H, PH, d, J = 96 Hz), 4.59 (2 H, AB quartet, J = 9.6 Hz), 4.46 (2 H, AB quartet, J = 7.2 Hz), 3.72 (3 H, s), 3.70 (3 H, s), 1.69 (3 H, s), 1.68 (3 H, s), 1.36 (3 H, s), 1.35 (3 H, s).

MS (probe, 100°): m/e Calcd. for $C_{16}H_{23}O_5{}^{31}P$: 326.1283. found: 326.1282 (68), 298 (78), 280 (32), 267 (21), 249 (32), 240 (30), 239 (50), 238 (94), 237 (100), 221 (29), 220 (36), 216 (24), 198 (36), 185 (88), 184 (42), 183 (23), 174 (32), 157 (70), 156 (43), 149 (29), 91 (25).

Preparation of t-butyldimethylsilyl ether 37

A solution of methyl hydroxysterpurate (8 mg), imidazole (6 mg) and t-butyldimethylchlorosilane (10 mg) in DMF (0.5 ml) was stirred at room temperature for 12 h. Ether (50 ml) was added and the solution was washed with water (three times), dried (Na_2SO_4) and evaporated at reduced pressure. Chromatography of the crude product (eluent benzene-ether (9:1)) gave t-butyldimethylsilyl ether 37 as an oil (10 mg).

IR (film): 3520, 1735, 1435, 1380, 1255, 1137, 1080 cm^{-1}.

^1H NMR ($CDCl_3$): δ 3.75 (2 H, AB quartet, J = 12 Hz), 3.70 (3 H, s), 2.85 (1 H, d, J_{gem} = 17 Hz), 3.60 (1 H, br. mult.), 2.27 (1 H, d, J_{gem} = 17 Hz), 1.65 (3 H, s), 1.34 (3 H, s), 0.93 (9 H, t-butyl group, s), 0.12 (6 H, 2XSiCH_3, s).

MS (probe, 150°): m/e Calcd. for $C_{22}H_{38}O_4Si$: 394.2539. found: 394.2542 (41), 366 (23), 337 (22), 309 (27), 262 (20), 249 (27), 248 (21), 245 (25), 189 (91), 185 (66), 175 (33), 174 (75), 75 (100), 73 (60).

Hydrolysis of methyl hydroxysterpurate (28)

Methyl hydroxysterpurate (35 mg) was dissolved in methanol (0.75 ml) and 20% NaOH in methanol-water (4:1, 1 ml) added. The solution was stirred at room temperature for 4 h followed by the removal of methanol under

reduced pressure. Water (3 ml) was added and the aqueous solution was washed with ether, then acidified (ice bath) to pH 3 with 6 M HCl. The acidic solution was extracted three times with methylene chloride (15 ml portions) and the combined extract was washed successively with water and brine, dried (Na_2SO_4) and evaporated to give the crude hydroxysterpuric acid (23 mg). Column chromatography (eluent chloroform-methanol (9:1)) of the crude product followed by crystallization from ether-Skellysolve B provided pure hydroxysterpuric acid (2, 8 mg) as white crystals, mp 60-63°.

IR (film): 3360, 1700, 1437, 1380, 1220, 1132, 1071, 1025 cm^{-1}.

^1H NMR ($CDCl_3$): δ 4.75 (3 H, br, exchangeable), 3.84 (2 H on C-13, AB quartet J = 12 Hz), 2.84 (1 H on C-11, d, J_{gem} = 18 Hz), 2.62 (1 H on C-8, br. mult.), 2.27 (1 H on C-11, d, J_{gem} = 18 Hz), 1.66 (3 H on C-12, s), 1.38 (3 H on C-15, s), 1.22 (1 H on C-7, dd, $J_{7,8}$ = 11 Hz, J_{gem} = 13 Hz).

MS (probe, 150°): m/e Calcd. for $C_{15}H_{22}O_4$: 266.1518. found: 266.1511 (29), 238 (50), 189 (100), 175 (51), 174 (68), 161 (33), 145 (20), 105 (23), 91 (39), 77 (21).

Isolation of hydroxysterpuric acid (2)

Preparative tlc of the crude acidic metabolites

(90 mg, two ptlc plates, triple elution with benzene-acetone-acetic acid (80:20:1)) gave an impure sample of hydroxysterpuric acid (20 mg). This was chromatographed (eluent chloroform-methanol (9:1)) to give hydroxysterpuric acid (13 mg) as a very viscous colorless oil. Crystallization of this material from ether-Skellysolve B provided hydroxysterpuric acid (3 mg) in crystalline form, identical in all respects (mp, IR, ^{1}H NMR, MS, tlc) with the hydrolysis product of methyl hydroxysterpurate.

Isolation of methyl hydroxysterpurate ethylidene acetal (39)

The crude acidic metabolites (1.4 g) were chromatographed (eluent benzene-acetone (39:1) to (19:1)) to give a solid material (43 mg) which was dissolved in ether (5 ml) and then diazomethane (10 ml, 0.3 M) added. After standing at room temperature for 15 min ether was removed under reduced pressure and the residue was subjected to preparative tlc (triple elution with benzene-ether (3:1)) followed by column chromatography (eluent Skellysolve B-ether (9:1)) to provide methyl hydroxysterpurate ethylidene acetal (39) as a colorless oil (12 mg).

IR (film): 1731, 1432, 1400, 1372, 1308, 1235, 1160, 1112 cm^{-1}.

^{1}H NMR ($CDCl_3$): δ 5.05 (1 H, acetal proton, q, J = 5 Hz), 4.00 (2 H on C-13, AB quartet, J = 12 Hz), 3.70 (3 H, OCH_3, s), 2.92 (1 H on C-11, d, J_{gem} = 17 Hz), 2.75 (1 H, dd, J's 9.5 and 10.5 Hz), 2.58 (1 H on C-8, br. mult.), 2.24 (1 H on C-11, d, J_{gem} = 17 Hz), 1.90-1.70 (3 H, complex), 1.60 (3 H on C-12, s), 1.52-1.42 (3 H, complex), 1.34 (3 H on C-15, s), 1.30 (3 H, acetal methyl, d, J = 5 Hz), 1.20 (1 H on C-7, $J_{7,8}$ = 10 Hz, J_{gem} = 12 Hz).

MS (probe, 150°): m/e Calcd. for $C_{18}H_{26}O_4$: 306.1831. found: 306.1830 (16), 278 (14), 175 (31), 174 (100), 121 (27).

Methyl hydroxysterpurate (28) from methyl hydroxysterpurate ethylidene acetal (39)

The acetal 39 (7 mg) was dissolved in THF (0.5 ml) and aqueous 3 M HCl (0.5 ml) added. The reaction mixture was stirred at room temperature for 3 h. Ether (30 ml) was added and the solution was washed with water (three times) and brine, then dried (Na_2SO_4) and evaporated at reduced pressure. Chromatography of the crude product (eluent benzene-ether (3:1)) gave methyl hydroxysterpurate (28, 4 mg), identical (IR, ^{1}H NMR, MS, tlc) with an authentic sample.

Preparation of ethylidene acetals 39 and 40

A solution of methyl hydroxysterpurate (30 mg), acet-

aldehyde (1.7 ml) and p-toluenesulfonic acid (1 mg) was stirred at room temperature for 1 h, then the solution was evaporated and the residue taken up in ether (50 ml). The ether solution was washed successively with satd. sodium bicarbonate, water (three times), and brine. After drying (Na_2SO_4) and evaporation of the solvent the crude product was chromatographed (eluent Skellysolve B-ether (93:7)) to provide a mixture of 39, 40, and acetaldehyde trimer (20 mg). Preparative tlc (triple elution with Skellysolve B-ether (4;1)) separated the more polar component (unnatural epimer, 40) which was further purified by chromatography (eluent Skellysolve B-ether (4:1)) as a colorless oil (3.5 mg). The less polar component obtained from ptlc (which consisted of the natural epimer 39 and acetaldehyde trimer) was subjected to ptlc (triple elution with benzene-ether (19:1)) to give the natural epimer 39 in fairly pure form. Column chromatography (eluent benzene-ether (19:1)) provided pure acetal 39 as a colorless oil (3 mg), identical (IR, NMR, MS, tlc) with authentic sample. The unnatural epimer 40 shows the following spectral properties.

IR (film): 1731, 1450, 1400, 1380, 1307, 1238, 1137 cm^{-1}.

^1H NMR ($CDCl_3$): δ 4.57 (1 H, acetal proton, q, J = 5 Hz), 3.80 (2 H on C-13, AB quartet, J = 10.5 Hz), 3.70 (3 H, s), 2.87 (1 H, d, J_{gem} = 17 Hz), 2.61 (1 H,

br. mult.), 2.28 (1 H, d, J_{gem} = 17 Hz), 1.56 (3 H, s), 1.33 (3 H, s), 1.25 (3 H, acetal methyl, d, J = 5 Hz).
MS (probe, 150°): m/e Calcd. for $C_{18}H_{26}O_4$: 306.1831. found: 306.1824 (21), 278 (18), 174 (100).

Isolation of sterpurene-3,12,14-triol

The crude neutral metabolites (0.52 g) were subjected to flash chromatography (eluent chloroform-methanol (19:1)) to give the crude triol 4 (75 mg). Further purification by column chromatography (eluent benzene-acetone (3:1)) provided a crystalline sample of 4 (42 mg) which was recrystallized from ether to give pure sterpurene-3,12,14-triol (4, 30 mg) as white needles, mp 146-148°.
IR (film): 3315, 1436, 1420, 1368, 1207, 1170, 1138, 1072, 1027, 998 cm^{-1}.
^1H NMR ($CDCl_3$): δ 4.22 (2 H on C-12, AB quartet, J = 12 Hz), 3.39 (2 H on C-14, AB quartet, J = 12 Hz), 2.86 (3 H, br., exchangeable), 2.62 (1 H on C-8, br. mult.), 2.57 (1 H on C-11, d, J_{gem} = 18 Hz), 2.24 (1 H, dd, J's 11 and 19 Hz), 2.10 (1 H on C-11, d, J =18 Hz), 2.04 (1 H, complex mult.), 1.68 (1 H, m), 1.48 (1 H, dd, J's 9.5 and 19 Hz), 1.22 (2 H, complex mult.), 1.21 (3 H, s), 1.10 (3 H, s), 0.91 (1 H on C-7, dd, $J_{7,8}$ = 11 Hz, J_{gem} = 13 Hz).
MS (probe, 150°): m/e Calcd. for $C_{15}H_{24}O_3$: 252.1725.

found: 252.1730 (3), 234 (46), 224 (49), 216 (20), 204 (24), 203 (67), 188 (94), 175 (100), 173 (36), 159 (54), 145 (25), 131 (22), 119 (27), 107 (20), 105 (44), 91 (40).

Acetylation of triol 4

Acetic anhydride (0.25 ml) was added to a solution of triol 4 (5 mg) in pyridine (0.5 ml). The reaction mixture was stirred at room temperature for 0.5 h, then toluene (5 ml) was added and the solvents were evaporated under reduced pressure. Column chromatography (eluent benzene-ether (9:1)) provided the diacetate 41 as a colorless oil (5 mg).

IR (film): 3455, 1737, 1450, 1422, 1365, 1929, 1130, 1021 cm^{-1}.

^1H NMR ($CDCl_3$): δ 4.74 (2 H on C-12, AB quartet, J = 12 Hz), 3.90 (2 H on C-15, AB quartet, J = 12 Hz), 2.68 (1 H on C-8, br. mult.), 2.42 (1 H on C-11, d, J_{gem} = 17 Hz), 2.25 (1 H on C-11, d, J_{gem} = 17 Hz), 2.20 (1 H on C-4, m), 2.08 (3 H, acetate methyl, s), 2.07 (1 H, m), 2.06 (3 H, acetate methyl, s), 1.71 (1 H on C-9, dd, $J_{8,9}$ = 12 Hz, J_{gem} = 12.5 Hz), 1.63 (1 H on C-7, dd, $J_{7,8}$ = 6 Hz, J_{gem} = 13 Hz), 1.55 (1 H on C-5, m), 1.30 (1 H on C-5, m), 1.26 (1 H on C-9, $J_{8,9}$ = 6 Hz, J_{gem} = 12.5 Hz), 1.24 (3 H on C-13, s), 1.14 (3 H on C-15, s), 0.88 (1 H on C-7, dd, $J_{7,8}$ = 11 Hz, J_{gem} = 13 Hz). The assignments of the coupling partners were confirmed by

double irradiation experiments.

^{1}H NMR (Py-$\underline{d}_5$): δ 5.20 (2 H on C-12, AB quartet, J = 12 Hz), 4.03 (2 H, AB quartet, J = 12 Hz), 2.71 (1 H, br. mult.), 2.06 (3 H, s), 1.55 (1 H, m), 1.42 (3 H on C-13, s), 1.32 (1 H, m), 1.12 (3 H, s), 0.84 (1 H, dd, J's 11 and 13 Hz).

Chemical Ionization (NH_3) MS shows the M^++18 (m/e) 354 peak).

MS (probe, 150°): the highest peak corresponded to M^+-H_2O. m/e Calcd. for $C_{19}H_{26}O_4$ (M^+-H_2O): 318.1831. found: 318.1851 (0.5), 308 (15), 276(20), 247 (21), 216 (41), 203 (33), 188 (100), 175 (55), 173 (44), 105 (21), 91 (20).

Sterpurene-3,12,14-triol acetonide (42)

Sterpurene-3,12,14-triol (7 mg) was dissolved in 2,2-dimethoxypropane(0.5 ml) and a catalytic amount of p-toluenesulfonic acid added. After stirring at room temperature for 2 h ether (50 ml) was added and the solution was washed successively with satd. sodium bicarbonate, water (three times), and brine. The solution was dried (Na_2SO_4) and evaporated to give the crude product which was purified by chromatography (eluent benzene-ether (19:1)) to provide acetonide 42 as a colorless oil (7 mg).

IR (film): 3385, 1453, 1378, 1240, 1192, 1087, 1030 cm^{-1}.

^{1}H NMR ($CDCl_3$): δ 4.33 (2 H on C-12, AB quartet, J = 12 Hz), 3.47 (2 H on C-14, s), 2.64 (1 H, br. mult.), 2.38 (1 H, d, J_{gem} = 17 Hz), 2.17 (1 H, m), 1.92 (1 H, d, J_{gem} = 17 Hz), 1.44 (3 H, s), 1.25 (3 H, s), 1.18 (3 H, s), 1.10 (3 H, s), 0.92 (1 H on C-7, dd, $J_{7,8}$ = 11 Hz, J_{gem} = 13 Hz).

MS (probe, 25°): m/e Calcd. for $C_{18}H_{28}O_3$: 292.2039. found: 292.2034 (0.7), 235 (27), 234 (100), 216 (29), 206 (38), 204 (39), 203 (89), 189 (21), 188 (78), 187 (33), 175 (41), 159 (27), 119 (23), 105 (35), 91 (41), 79 (20), 77 (20), 55 (23).

Isolation of lactone "A" (6) and lactone "B" (7)

Ether (40 ml) was added to the crude neutral metabolites (2.2 g) and the solution was warmed to dissolve the oily material. The resulting suspension was kept at -12° for several days. This was then filtered to provide a white solid containing lactone "A", lactone "B", and some minor compounds (a total of 60 mg). This mixture was subjected to column chromatography over silica gel. Elution with 5-15% ether in benzene gave lactone "A" (6, 8 mg), and elution with 20-30% ether in benzene provided lactone "B" (7, 34 mg) both of which were obtained as white solids.

Lactone "A" (6)

The crude lactone "A" (8 mg), eluted from the

column as the less polar component, was recrystallized from the ether and a few drops of acetone to provide pure lactone "A" (6, 7 mg) as white needles, mp 228-232°.

IR (film): 1771, 1701, 1650, 1439, 1403, 1361, 1327, 1230, 1193, 1118, 1105, 1064, 1000, 970, 938 cm^{-1}.

UV (CH_3OH): λ_{max} = 239 nm.

^1H NMR ($CDCl_3$): δ 5.72 (1 H on C-4, s), 4.55 (1 H on C-12, dd, $J_{3,12}$ = 8 Hz, J_{gem} = 8 Hz), 3.66 (1 H on C-3, dd, J = 8 Hz), 3.52 (1 H on C-12, dd, $J_{3,12}$ = 8 Hz, J_{gem} = 8 Hz), 2.95 (2 H on C-8, AB quartet, J = 20 Hz), 2.43 (2 H on C-10, AB quartet, J = 18 Hz), 1.60 (2 H on C-5, AB quartet, J = 6 Hz), 1.14 (3 H, s), 1.13 (3 H, s). The assignments of coupling partners were confirmed by double irradiation experiments.

^{13}C NMR ($CDCl_3$): δ 210.8 (C-1, s), 175.1 (C-13, s), 165.4 (C-9, s), 132.7 (C-2, s), 105.4 (C-4, d), 74.9 (C-12, t), 46.6 (t), 44.5 (s), 40.6 (s), 31.7 (d), 28.1 (t), 25.1 (2Xq), 24.5 (s), 19.9 (C-5, t).

MS (probe, 250°): m/e Calcd. for $C_{15}H_{16}O_4$: 260.1059. found: 260.1049 (77), 232 (35), 231 (47), 230 (100), 202 (42), 187 (55), 174 (22), 173 (35), 159 (53), 145 (20), 131 (33), 91 (20).

Preparation of tetracetate 45

Lactone "A" (6, 4.5 mg) was dissolved in THF (1 ml)

and lithium aluminum hydride (15 mg) added. The reaction mixture was stirred at room temperature for 20 min., then excess hydride was destroyed by the addition of wet ether. After filtration and evaporation of the solvents under reduced pressure chloroform (30 ml) was added and the resultant solution was washed with water (three times), then with brine. Drying (Na_2SO_4) and evaporation of the solvent gave an oil (3 mg) which was dissolved in pyridine (0.5 ml) and acetic anhydride (0.3 ml) added. The mixture was stirred at room temperature for 15 h, then toluene (5 ml) was added and the solvents were evaporated. Chromatography of the crude product (eluent benzene-ether (9:1)) provided pure tetraacetate 45 as an oil (0.8 mg).

IR (film): 1735, 1623, 1457, 1372, 1360, 1223 cm^{-1}.

^1H NMR ($CDCl_3$): δ 5.33 (1 H on C-1, s), 4.40-4.00 (6 H, three methylene groups geminal to the primary acetoxyl groups, complex), 2.86 (1 H on C-3, br. mult.) 2.44 (2 H on C-8, AB quartet, J = 16 Hz), 2.06 (2 H on C-10, AB quartet, J = 16 Hz), 2.06 (6 H, acetate methyls, 2 s), 2.04 (3 H, acetate methyl, s), 2.02 (3 H, acetate methyl, s), 1.08 (3 H, s), 0.93 (3 H, s), 0.66 (2 H on C-5, AB quartet, J = 5 Hz).

Chemical Ionization (NH_3) MS shows M^++18 (m/e 454) peak.

MS (prob, 150°): The highest peak corresponded to $M^+-C_2H_4O_2$. m/e Calcd. for $C_{21}H_{28}O_6$ ($M^+-C_2H_4O_2$): 376.1885.

found: 376.1889 (6), 316 (73), 274 (38), 256 (79), 243 (42), 241 (25), 214 (58), 201 (45), 196 (100), 184 (23), 183 (79), 181 (25), 91 (20).

Lactone "B" (7)

The crude lactone "B" (7, 34 mg) eluted as the more polar component from the chromatography of the precipitate obtained from the neutral fraction (see above) was recrystallized from ether to provide the pure lactone "B" (25 mg) as fine needles, mp 210-212°

IR (film): 3450, 1760, 1695, 1640, 1431, 1397, 1331, 1238, 1176, 1108, 1092, 1043, 985 cm^{-1}.

UV (CH_3OH): λ_{max} = 238 nm.

^1H NMR ($CDCl_3$): δ 4.48 (2 H on C-4, AB quartet, J = 9 Hz), 3.88 (1 H on C-12, dd, $J_{3,12}$ = 3.5 Hz, J_{gem} = 11 Hz), 3.70 (1 H on C-12, dd, $J_{3,12}$ = 5 Hz, J_{gem} = 11 Hz), 3.18 (1 H on C-3, br. mult.), 2.88 (2 H on C-8, AB quartet, J = 19.5 Hz), 2.42 (2 H on C-10, AB quartet, J = 18 Hz), 2.06 (1 H, OH, exchangeable), 1.10 (3 H, s), 1.09 (3 H, s), 1.06 (2 H on C-5, AB quartet, J = 5 Hz). The assignments of coupling partners were verified by double irradiation experiments.

MS (probe, 150°): m/e Calcd. for $C_{15}H_{18}O_4$: 262.1205. found: 262.1208 (14), 233 (25), 232 (100), 188 (24).

Acetylation of lactone "B" (7)

Acetic anhydride (0.75 ml) was added to a solution

of lactone "B" (7 mg) in pyridine (1.5 ml) and the mixture was stirred at room temperature for 3 h. Toluene (5 ml) was added and the solvents were evaporated under reduced pressure. Preparative tlc of the crude product (benzene-ether (1:1)) gave acetate 46 (6 mg) which was subjected to column chromatography (eluent benzene-ether (4:1)) to give 46 as white crystals, mp 168-169°.

IR (film): 1755, 1690, 1645, 1453, 1418, 1398, 1376, 1332, 1242 cm^{-1}.

^1H NMR ($CDCl_3$): δ 4.34 (2 H, AB quartet, J = 10 Hz), 4.29 (1 H on C-12, $J_{3,12}$ = 5 Hz, J_{gem} = 10 Hz), 4.26 (1 H on C-12, $J_{3,12}$ = 3.5 Hz, J_{gem} = 10 Hz), 3.37 (1 H on C-3, br. mult.), 2.87 (2 H, AB quartet, J = 19.5 Hz), 2.40 (2 H, AB quartet, J = 18.5 Hz), 2.00 (3 H, acetate methyl, s), 1.11 (3 H, s), 1.10 (2 H, AB quartet J = 5 Hz), 1.08 (3 H, s).

Preparation of tetraacetates 45 and 47 from lactone "B" (7)

Lactone "B" (7, 4 mg) was reduced with lithium aluminum hydride and this was followed by acetylation of the crude product using the same procedure described for reduction-acetylation of lactone "A" (6). Chromatography of the crude tetraacetate (eluent benzene-ether (9:1)) gave a 1:1 mixture of 45 and 47 (total

weight 0.7 mg).

IR (film): 1735, 1460, 1372, 1360, 1223 cm^{-1}.

^1H NMR ($CDCl_3$): δ 5.35 (s), 5.33 (s), 4.40-4.00 (6 H, three methylene groups geminal to the primary acetoxyl groups, very complex), 2.86 (br. mult.), 2.76 (br. mult.), 2.06 (3 H, s), 2.05 (3 H, s), 2.04 (3 H, s). 2.02 (3 H, s), 1.08 (methyl singlet), 1.04 (methyl singlet), 0.66 (2 H, AB quartet, J = 5 Hz).

Chemical ionization (NH_3) MS shows M^++18 (m/e 454) peak.

MS (probe, 150°): the highest peak corresponded to $M^+-C_2H_4O_2$. m/e Calcd. for $C_{21}H_{28}O_6$ ($M^+-C_2H_4O_2$): 376.1886. found: 376.1915 (4), 316 (64), 274 (34), 256 (80), 2343 (35), 241 (27), 214 (60), 201 (40), 196 (100), 185 (21), 183 (76), 181 (26), 91 (20).

Extraction of the mycelium of S. purpureum and the isolation of l-sterpurene (5)

The mycelium of 10 ℓ growth of S. purpureum was kept at -12° for three months. It was then extracted with chloroform for 2 days in a Soxhlet apparatus. The chloroform extract was washed with water and brine, dried (Na_2SO_4), and concentrated under reduced pressure to provide the crude mycelium extract as a dark brown oil (0.62 g). Skellysolve B (10 ml) was added to the crude extract and the mixture was heated at 60° for 5 min. After cooling to room temperature, the Skellysolve

B portion was triturated and evaporation of the solvent gave the Skellysolve B extract as a yellow oil (0.35 g). The latter was subjected to column chromatography (eluent Skellysolve B) to provide 1-sterpurene as a colorless oil (12 mg).

IR (film): 1457, 1445, 1433, 1372, 1361, 1355 cm^{-1}.

^1H NMR ($CDCl_3$): δ 2.65 (1 H on C-8, br. mult.), 2.39 (1 H on C-3, dd, J's 10.5 and 13.5 Hz), 2.16 (1 H, m), 2.10 (2 H on C-11), 1.94 (1 H, m), 1.68 (1 H on C-9, dd, $J_{8,9}$ = 7.5 Hz, J_{gem} = 12 Hz), 1.58 (1 H on C-7, m), 1.53 (3 H on C-12, s), 1.48 (1 H, m), 1.44 (1 H, m), 1.23 (3 H, s), 1.10 (3 H, s), 0.94 (1 H, m), 0.69 (1 H on C-7, dd, $J_{7,8}$ = 11 Hz, J_{gem} = 13 Hz). The assignments of most of the coupling partners were verified by double irradiation experiments.

MS (probe, 100°): m/e Calcd. for $C_{15}H_{24}$: 204.1878. found: 204.1878 (34), 176 (100), 175 (82), 161 (81), 149 (24), 121 (37), 120 (41), 119 (43), 111 (26), 107 (42), 105 (54), 97 (32), 95 (29), 93 (32), 91 (32), 85 (28), 83 (30), 81 (29), 79 (22), 71 (35).

Epoxidation of 1-sterpurene

m-Chloroperbenzoic acid (10 mg of 85%) was added to an ice cold solution of 1-sterpurene (5, 7 mg) in methylene chloride (0.8 ml). The reaction mixture was left to stand in the fridge for 1 h, then an additional

0.5 h at room temperature. Ether (25 ml) was added and the resultant solution was washed successively with satd. sodium bicarbonate, water (two times), and brine. Drying (Na_2SO_4) and evaporation of the solvents gave the crude product (7 mg) which was purified by chromatography (eluent Skellysolve B) to provide epoxide 20 as a colorless oil (4 mg).

IR (film): 1600, 1455, 1445, 1432, 1363, 1355, 1280, 1188 cm^{-1}.

^1H NMR ($CDCl_3$): δ 2.45 (1 H, br. mult.), 2.14 (2 H, m), 1.95 (1 H, d, J = 15 Hz), 1.85 (1 H, br. mult.), 1.67 (1 H, dd, J's 7 and 12 Hz), 1.60-1.30 (6 H, complex), 1.19 (3 H, s), 1.18 (3 H, s), 1.15 (3 H, s), 1.12 (3 H, s).

MS (probe, 100°): m/e Calcd. for $C_{15}H_{24}O$: 220.1827. found: 220.1819 (24), 219 (22), 163 (21), 149 (91), 95 (100), 57 (65), 55 (33).

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APPENDIX

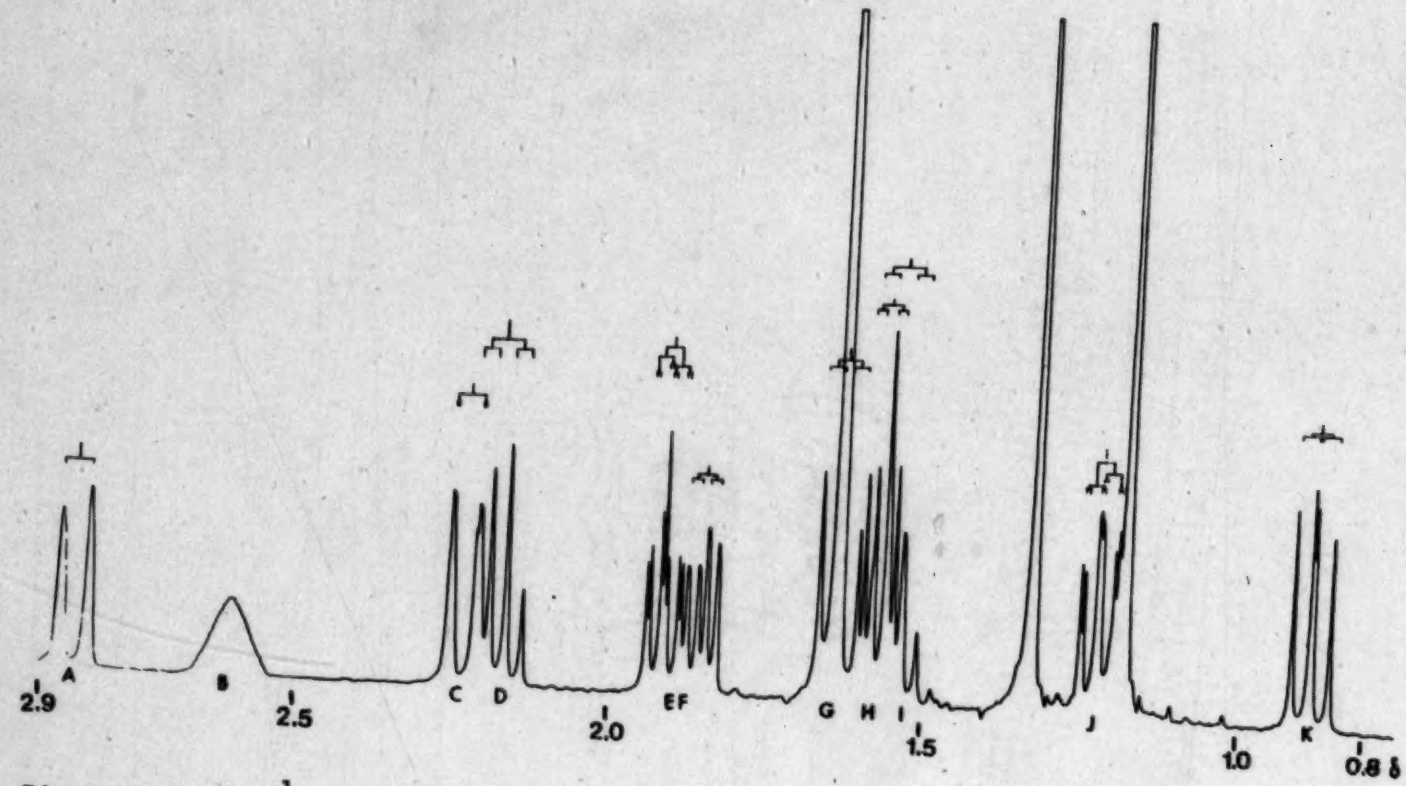


Figure 1. The ^1H nmr spectrum of sterpuric acid (1).

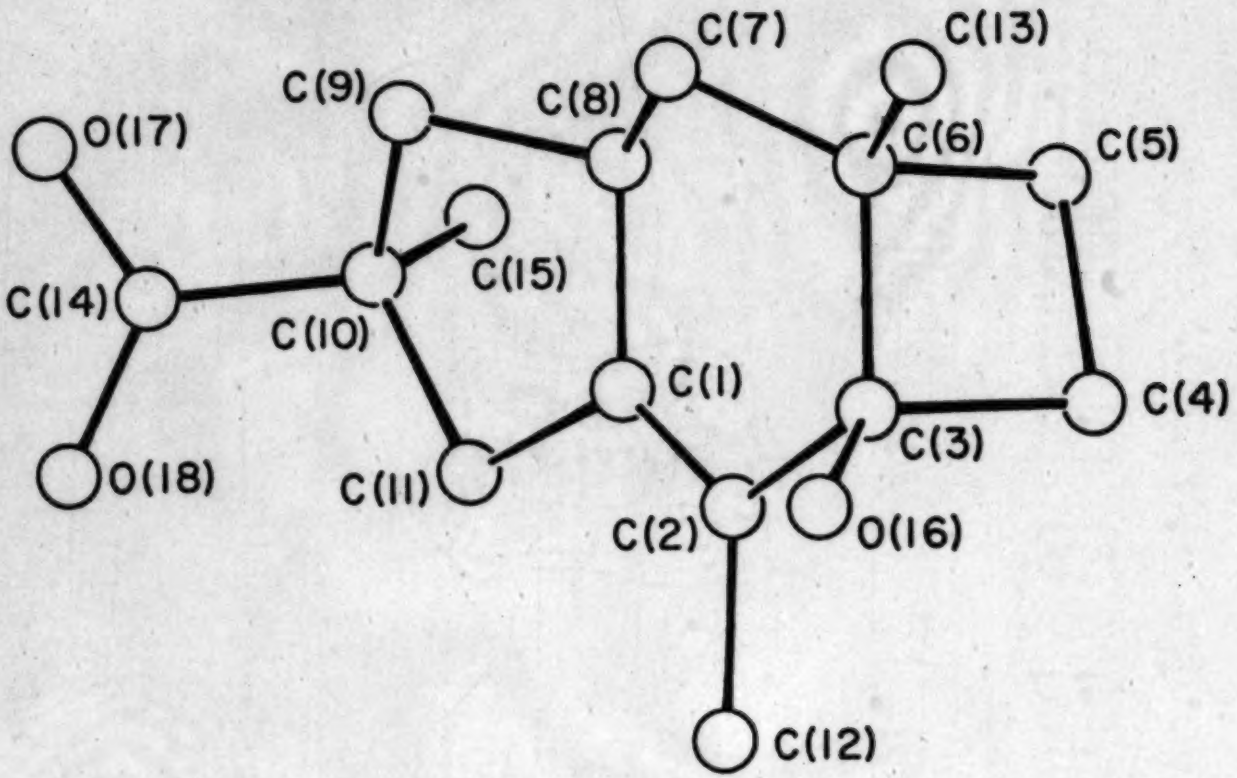


Figure 2. The computer generated perspective drawing of the x-ray structure of sterpuric acid (1).

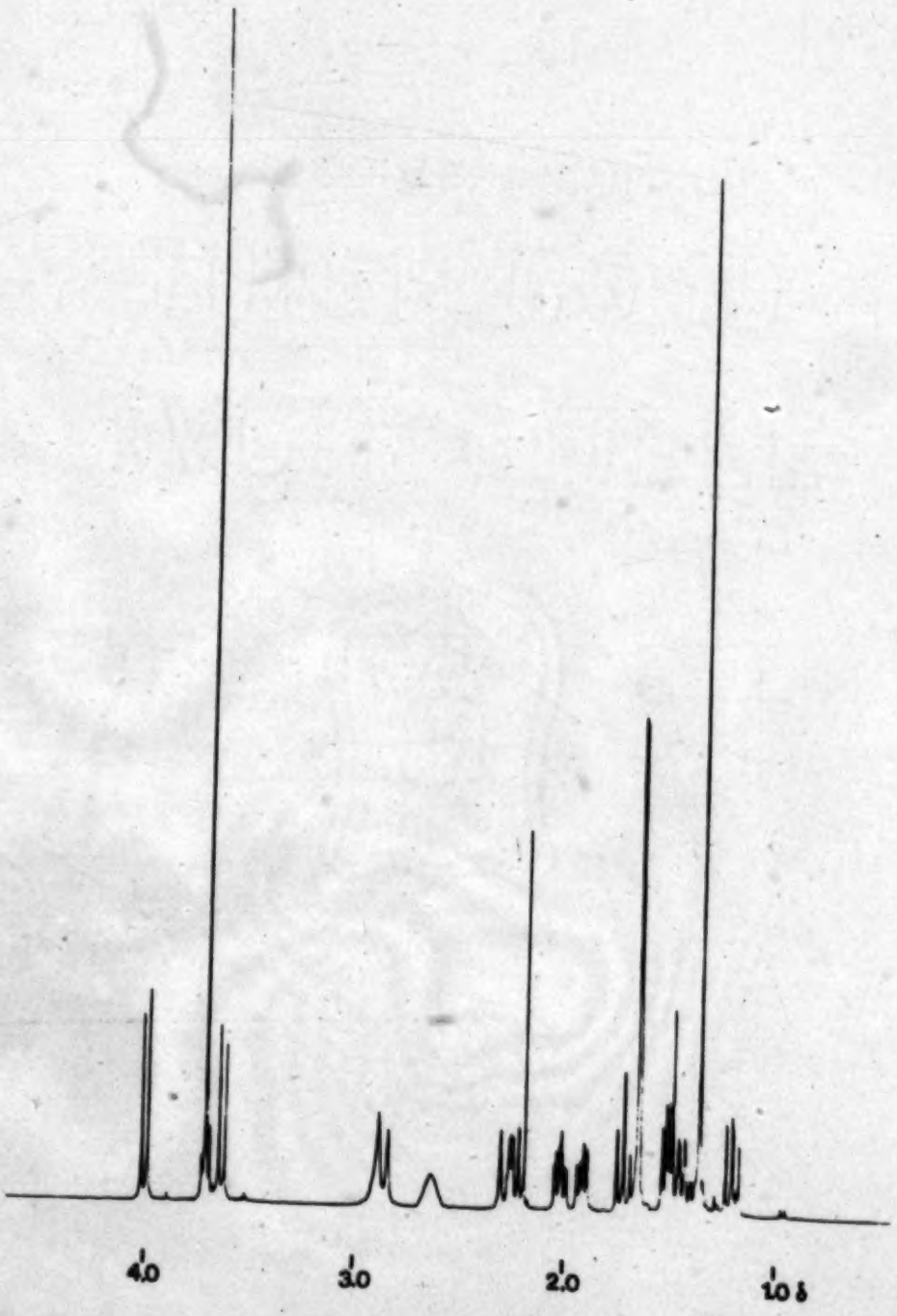


Figure 3. The ^{1}H nmr spectrum of methyl hydroxysterpurate (28).

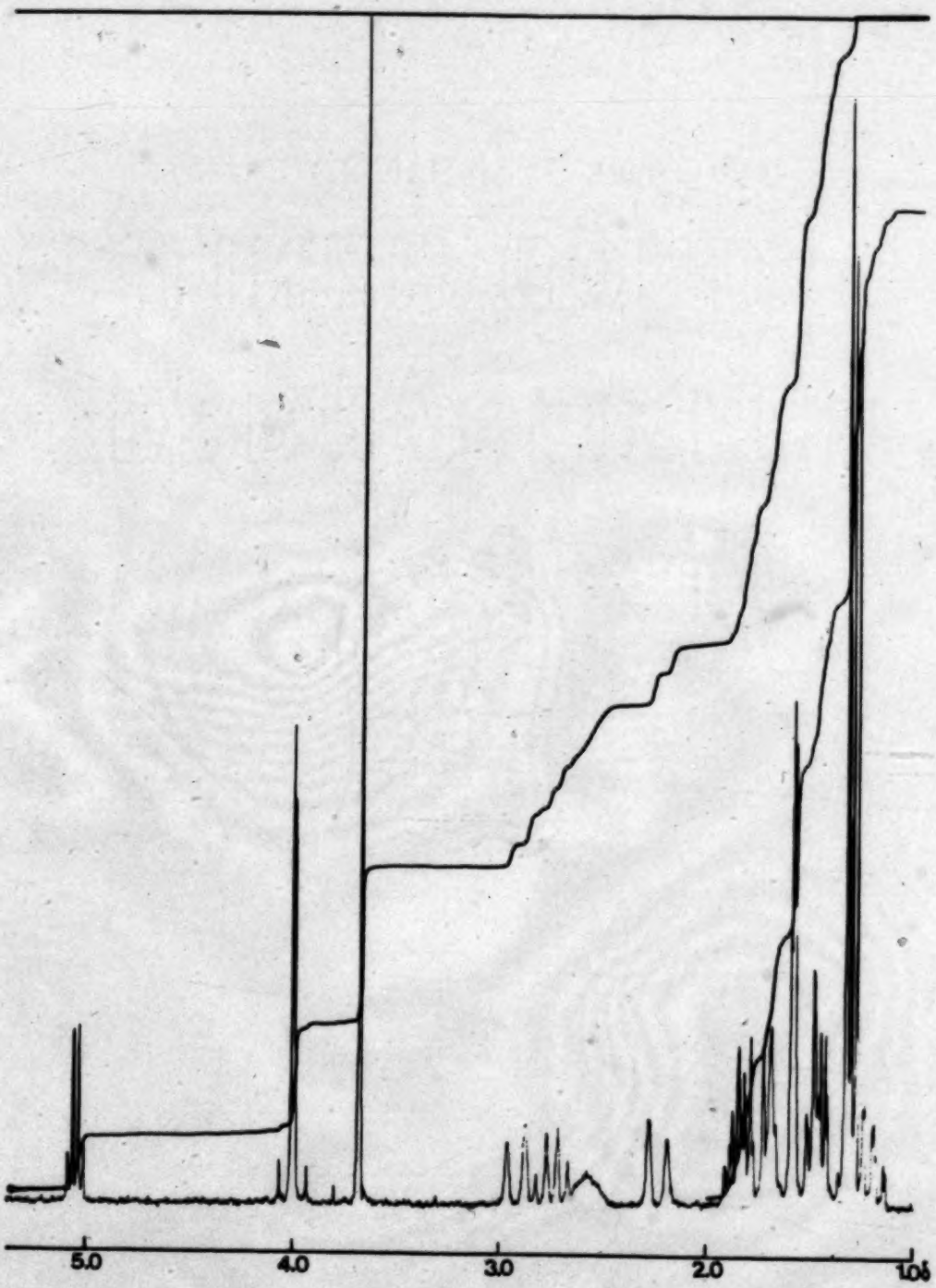


Figure 4. The ^1H nmr spectrum of methyl hydroxysterpurate ethylidene acetal (39).

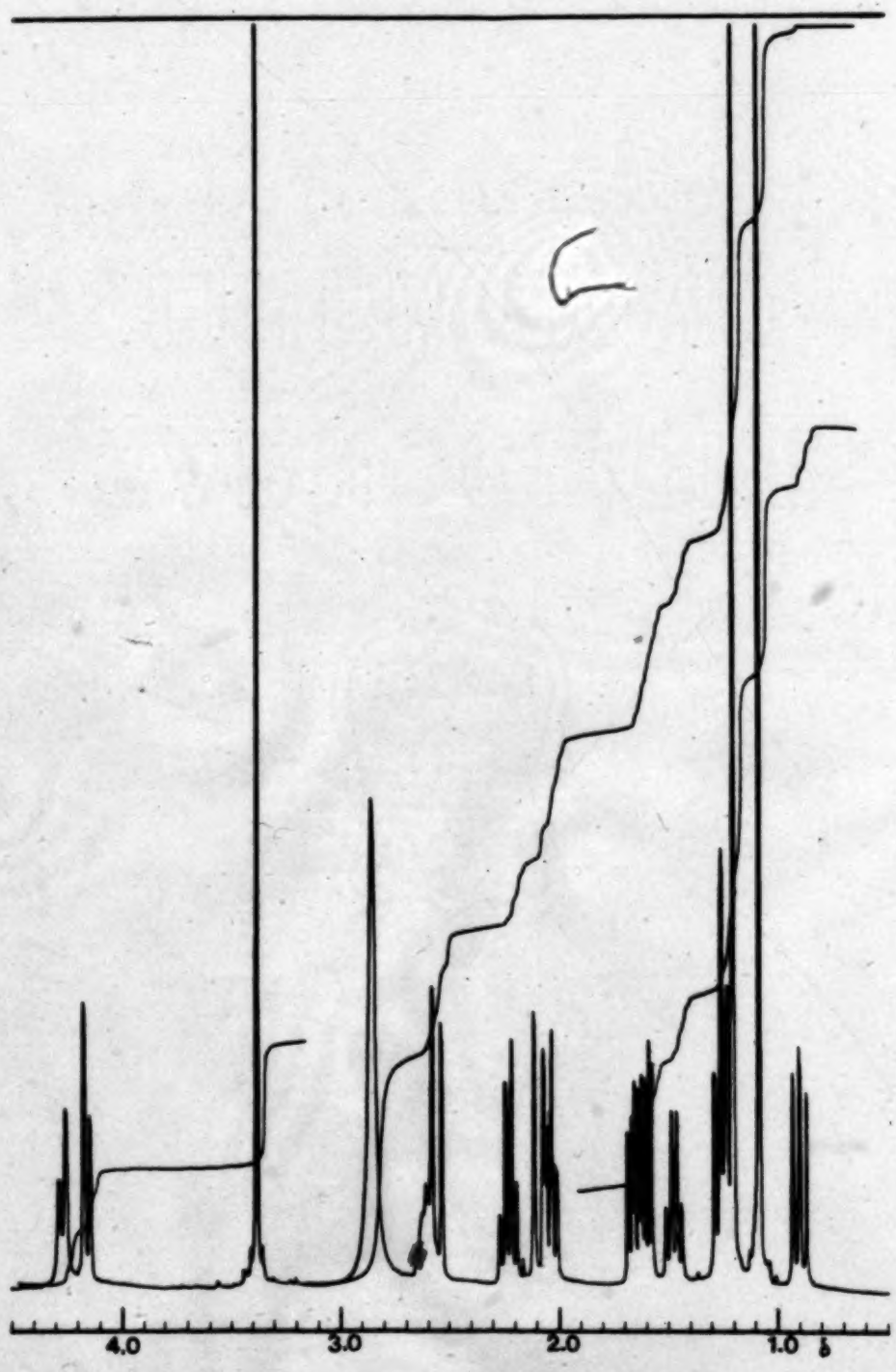


Figure 5. The ^{1}H nmr spectrum of sterpurene-3,12,14-triol (4).

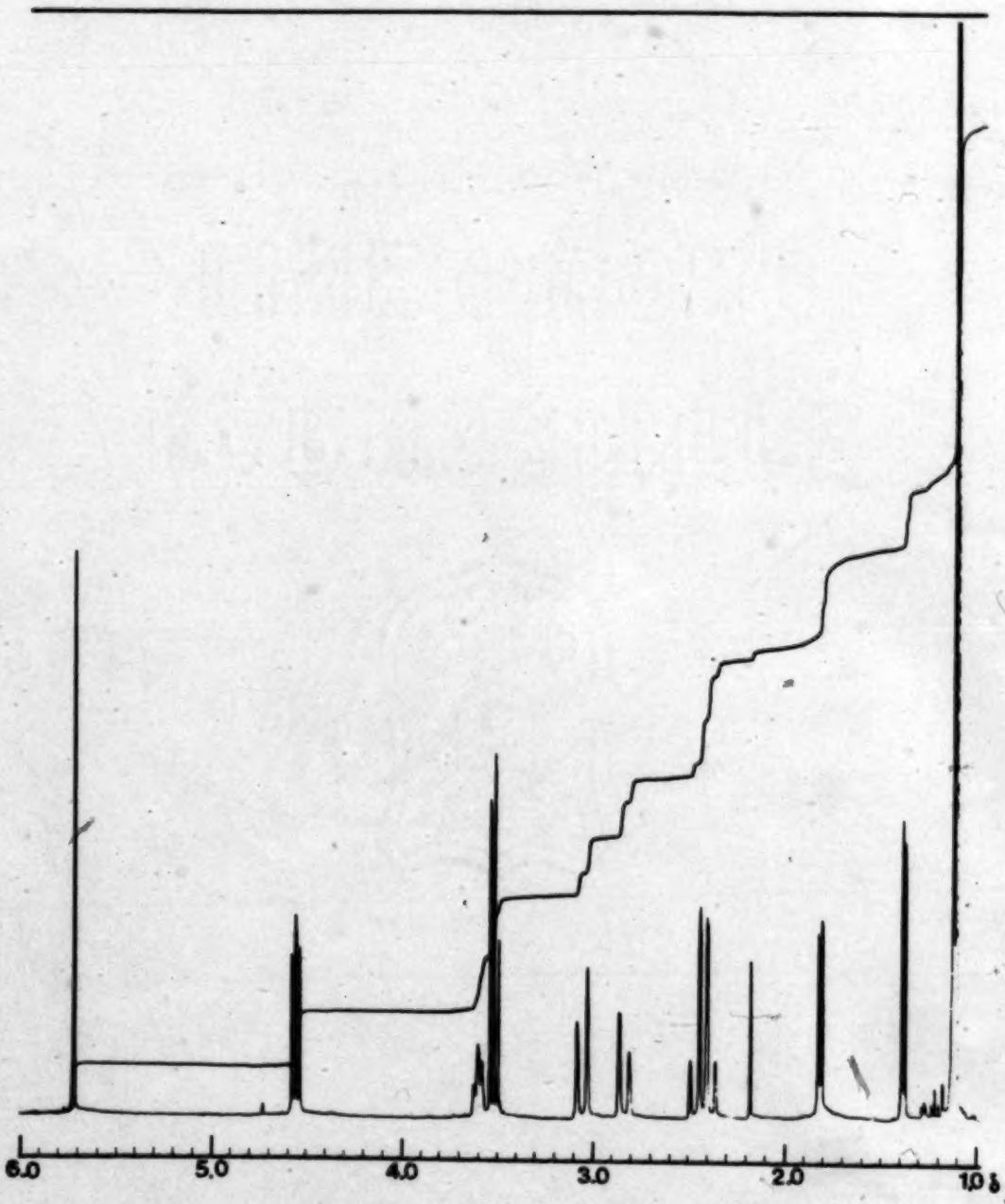


Figure 6. ^{1}H nmr spectrum of lactone "A" (6).

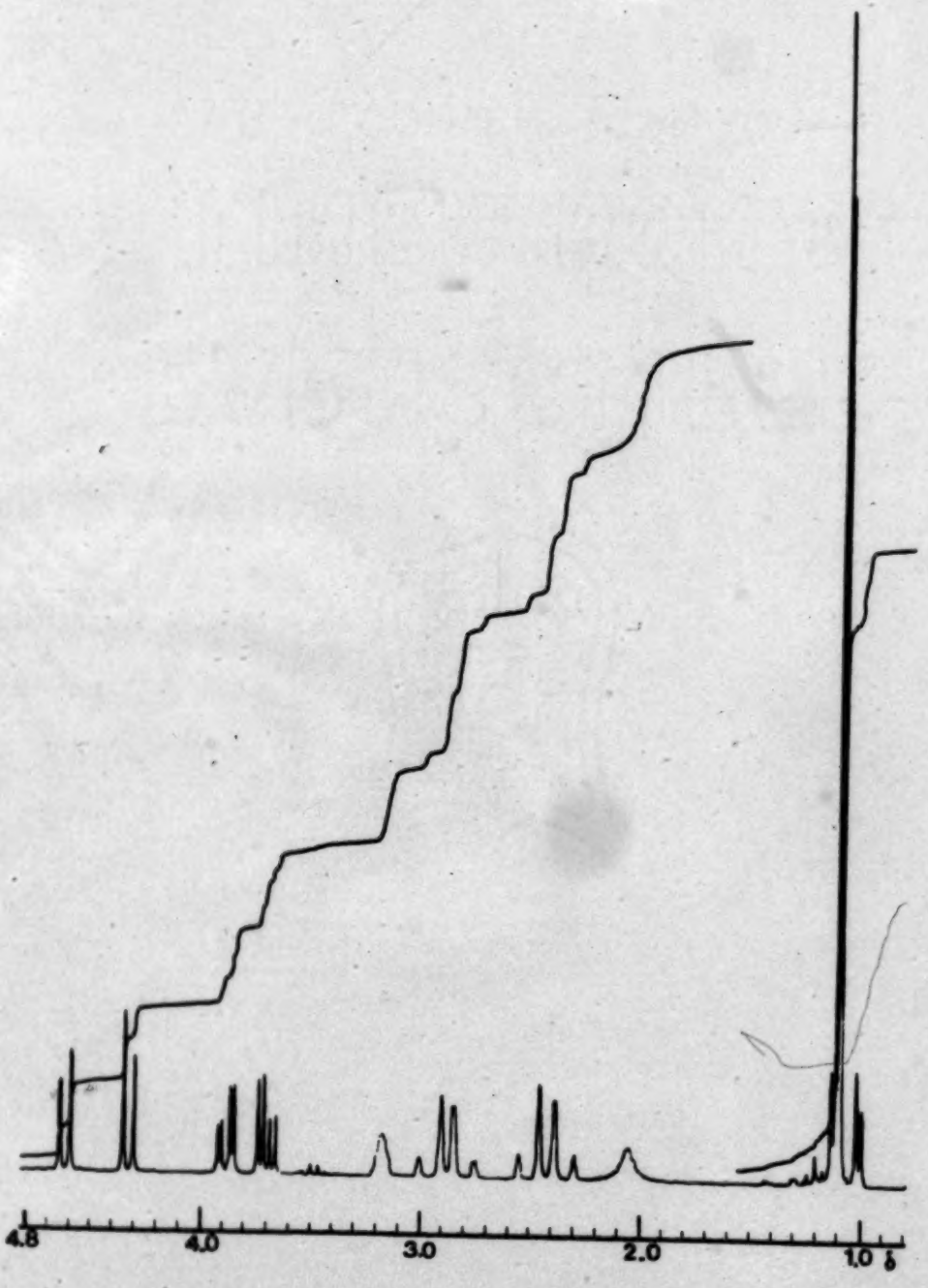


Figure 7. ^{1}H nmr spectrum of lactone "B" (7).

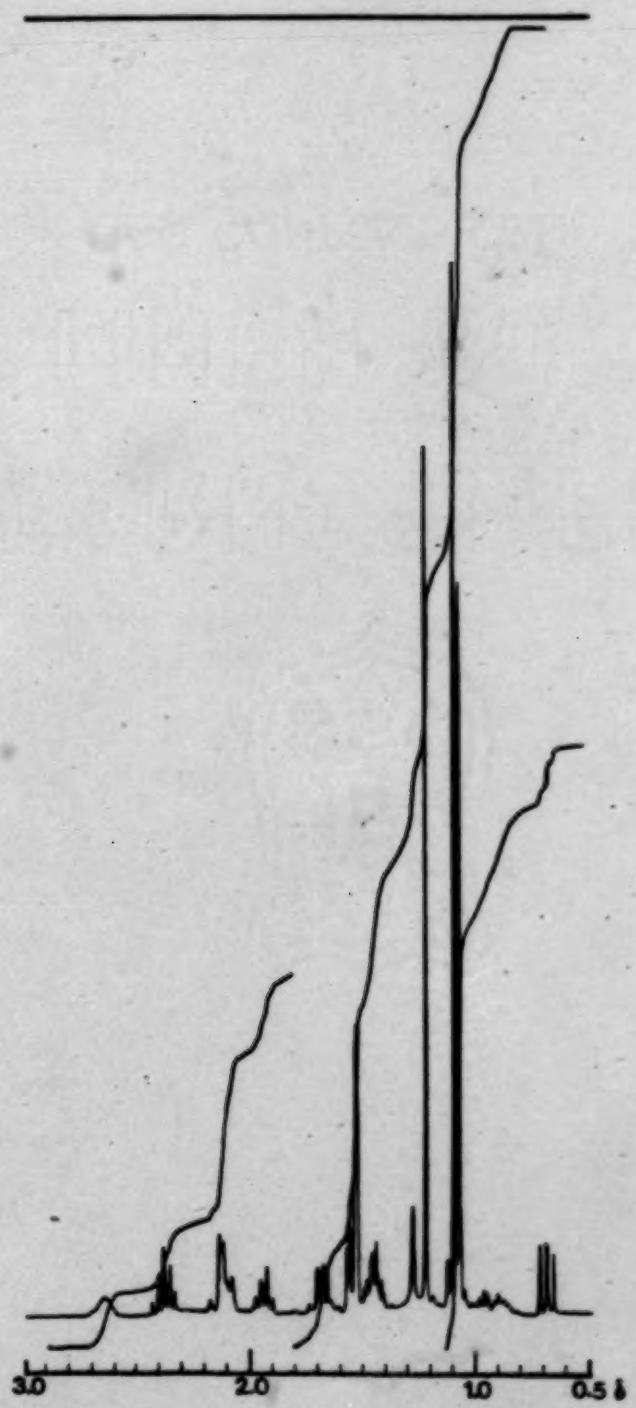


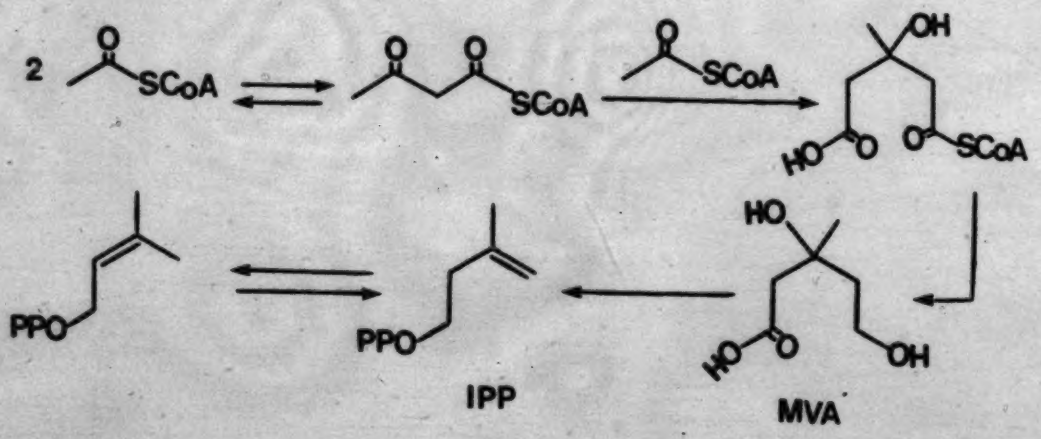
Figure 8. ^{1}H nmr spectrum of sterpurene (5).

II: STUDIES ON THE BIOSYNTHESIS OF STERPURANES

INTRODUCTION

A biosynthetic mechanism, like its chemical counterpart, can never be proved; it is only possible to provide data that are consistent with a proposed scheme and, hopefully, are exclusive of all other schemes devised to date. It seems that many biochemists and natural product chemists have much to learn on this point from physical chemists.[1]

According to current biosynthetic theory as developed by Ruzicka, Eschenmoser, Arigoni, and Hendrickson,[2] 2-trans-6-trans-farnesyl pyrophosphate (1) or the corresponding 2-cis-6-trans-compound 2 is regarded as the biological precursor of almost all sesquiterpenes. The formation of 2 could involve direct biosynthesis from mevalonic acid or from 2-trans-6-trans isomer (1) via nerolidol which is also a naturally occurring compound. Farnesyl pyrophosphate (FPP) is formed from acetyl coenzyme A via mevalonic acid (MVA), isopentenyl pyrophosphate (IPP), and geranyl pyrophosphate (GPP) as shown below.



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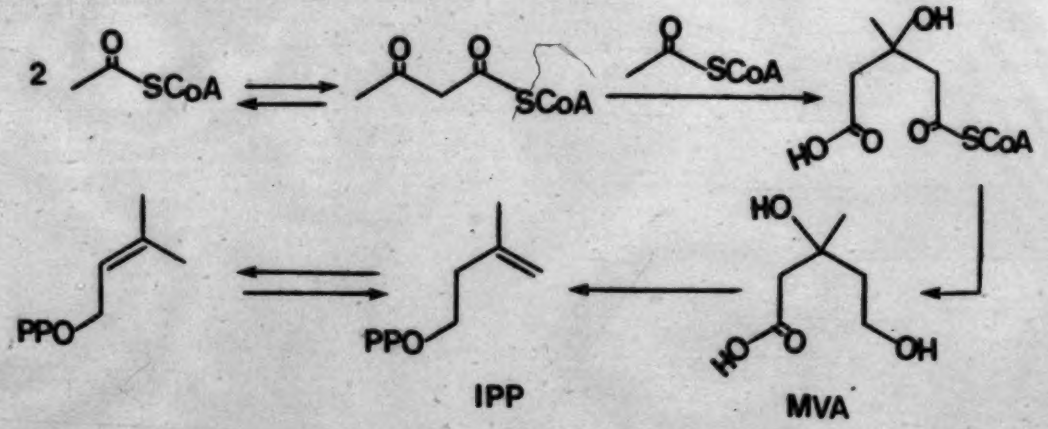
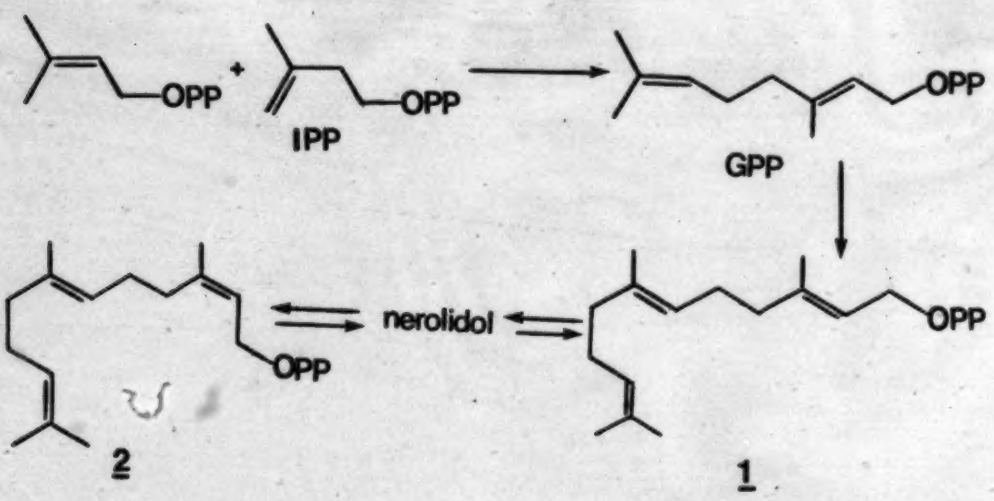
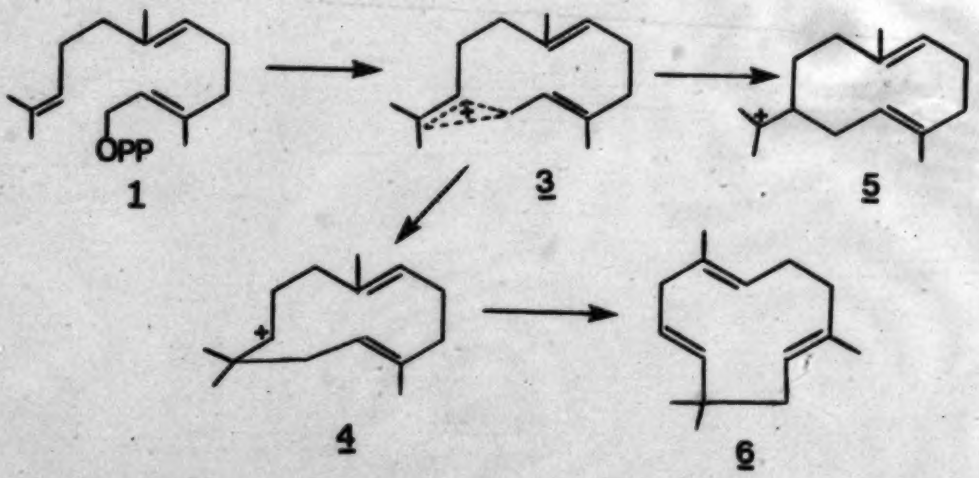
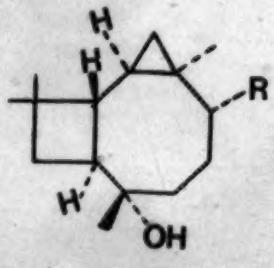




Removal of the pyrophosphate group from 2-trans-6-trans-FPP (1) yields a carbocation whose formulation as the nonclassical cation 3 explains its cyclization to two cyclic cations 4 and 5.[3] Deprotonation of the carbocation 4 affords the 11-membered ring hydrocarbon, humulene (6), which is the parent intermediate in the biosynthesis of a variety of sesquiterpenes.



Humulene (6) is a naturally occurring constituent of numerous essential oils.[4] Sutherland[5] and co-workers treated humulene (6) with N-bromosuccinimide in aqueous acetone to obtain the bromohydrin (7, R = Br). The latter could be converted stereospecifically, in two steps, into the naturally occurring caryophyllene (8) or back into humulene with retention of configuration. Furthermore, hydrolysis of 7 (R = Br) gave tricyclohumuladiol (7, R = OH) which was shown one year later to be a naturally occurring metabolite of Japanese hop oil.[6] McKervey and Wright[8] obtained the same diol (7, R = OH) by acid catalyzed (20% H_2SO_4) rearrangement of humulene epoxide (9), a known natural product.[7] On the basis of their findings and the fact that both caryophyllene (8) and humulene (6) can be derived form the bromohydrin (7, R = Br) by two *in vitro* steps, the above workers postulated that humulene and humulene epoxide may be involved in the biosynthesis of tricyclohumuladiol (7, R = OH) and caryophyllene (8).



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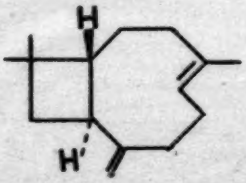

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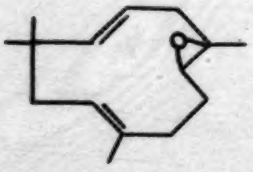
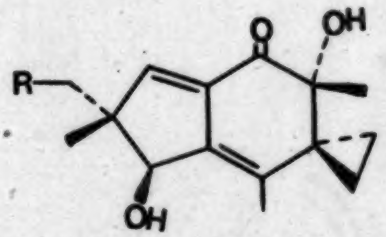


9

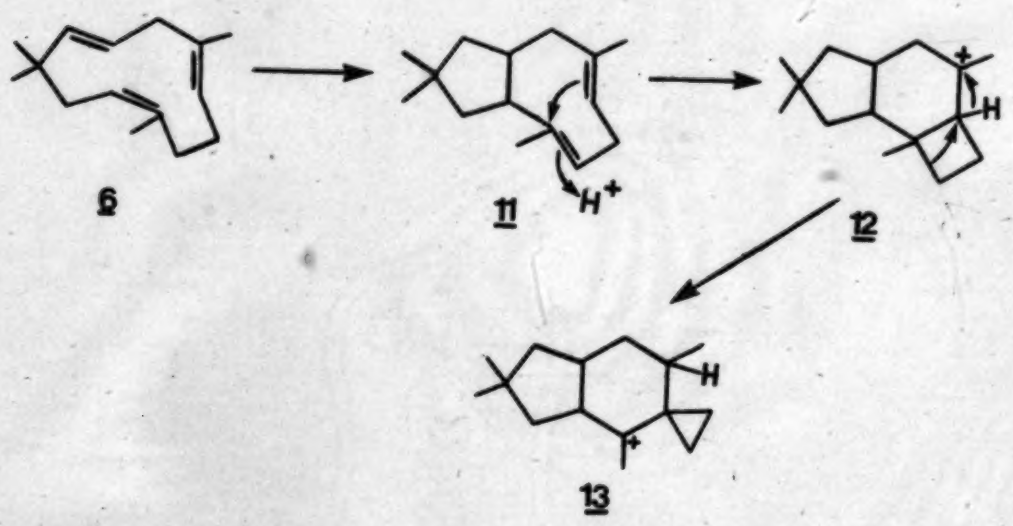
The discovery of illudin-M (10, R = H) and illudin-S (10, R = OH) in 1963 raised the possibility of involvement of humulene (6) in the biosynthesis of a variety of polycyclic sesquiterpenoids with novel skeletons.[9] The latter compounds were isolated from the culture liquid of the Basidiomycetes fungus Clitocybe illudens.



10

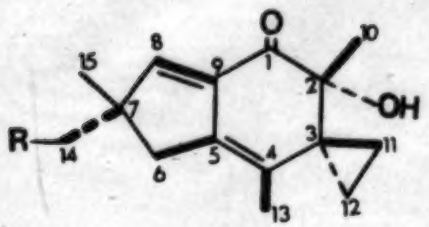
In their original publication disclosing the structural elucidation of illudin-M and -S, McMorris and Anchel proposed a possible biogenetic route in which humulene (6) is initially cyclized to the then unknown protoilludane cation (12). The latter subsequently undergoes a 1,2-hydride shift to afford the illudane cation (13). This proposal prompted Hanson and coworkers to undertake an extensive study of the detailed biogenesis of the illudane skeleton using a variety of singly- or doubly labeled precursors.[10] Thus, by the study of the incorporation of various tritium- and ^{14}C-labeled mevalonates these authors concluded that humulene (6) is cyclized first to the [6.3.0]

bicyclic system 11 in a non-concerted manner. A second cyclization provides the protoilludane cation (12) which in turn undergoes a 1,2-hydride shift and alkyl rearrangement to provide illudane cation (13). Deprotonation of the latter affords illudin skeleton.



Furthermore, the coupling pattern observed in illudin-M and -S derived from [1,2-$^{13}C_2$] acetate (see 14) and the induced coupling observed between C-6 and C-7 when the above compounds were obtained from [1-^{13}C] acetate is compatible with the proposed biosynthesis from FPP via the protoilludane cation (12). It should also be pointed out that the involvement of the postulated protoilludane cation (12) in the biosynthesis of illudins was supported further by the isolation of illudol[11] (15) and neoilludol[12] (product of allylic rearrange-

ment of illudol) from the same source.



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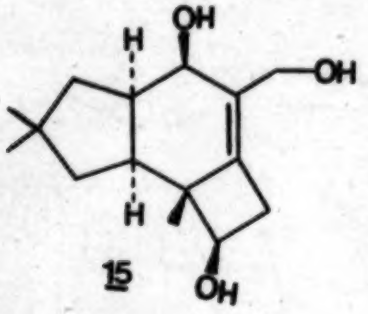
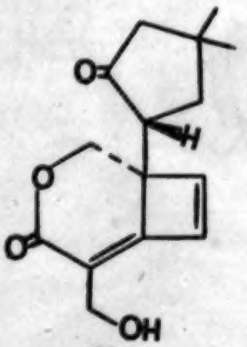


15

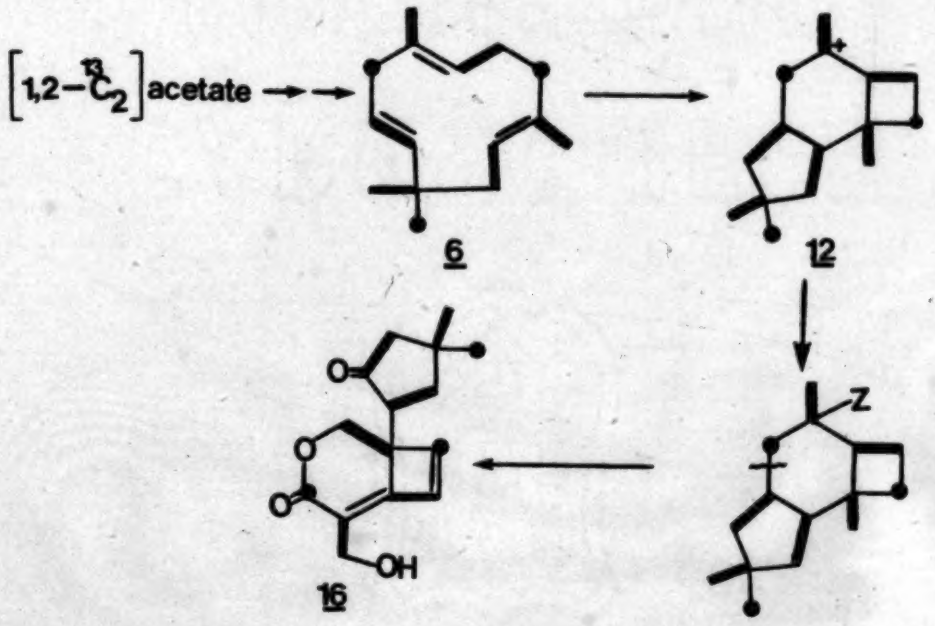
Fomannosin (16) is a biologically active sesquiterpene metabolite of the wood-rotting fungus Fomes annosus.[13] The novel ring structure of this unusual sesquiterpene is of special interest, since this is the first reported example of a sesquiterpene containing a cyclobutene moiety.



16

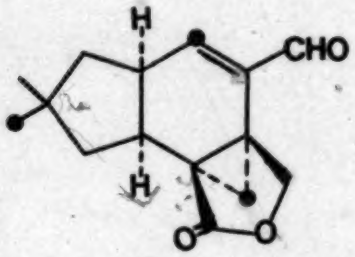
Fomannosin obeys the isoprene rule, but the isoprene units are not connected in the usual head-to-tail sequence. Although biogenetic speculation had centered on protoilludane type precursor, this assumption did not have any experimental support until 1976. Cane

and Nachbar undertook an isotope labelling study using [1,2-$^{13}C_2$] acetate.[14] Analysis of the ^{13}C nmr spectrum of the enriched fomannosin (16) provided very good evidence for the intermediacy of the protoilludane cation (12) in the biosynthesis of fomannosin as shown.



The structure of the antibacterial compound marasmic acid produced by the fungus Marasmius conigenus was deduced by de Mayo and co-workers to be 17.[15] These workers also investigated the biogenesis of this novel sesquiterpene. [2-^{14}C] MVA was incorporated into marasmic acid to the extent of 0.3%. Kuhn-Roth oxidation of the labeled compound indicated that marasmic acid (17) had one-third of the label at the geminal dimethyl

group and degradation of the ester 18 showed that about one-third of total activity resided in the cylcopropane methylene of marasmic acid. Although the detailed biosynthetic scheme was not elucidated, these workers suggested a biogenetic scheme for 17 involving the protoilludane precursor (12) and the cyclopropyl carbinyl cation 19 (marasmane skeleton) shown below.



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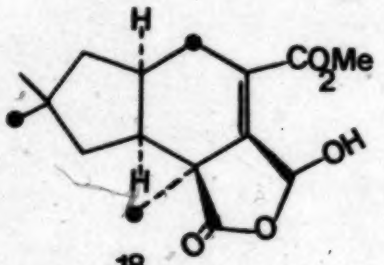

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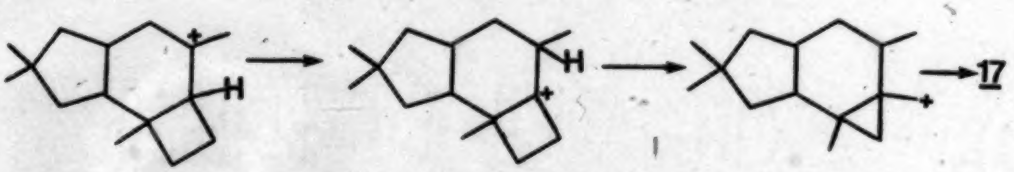
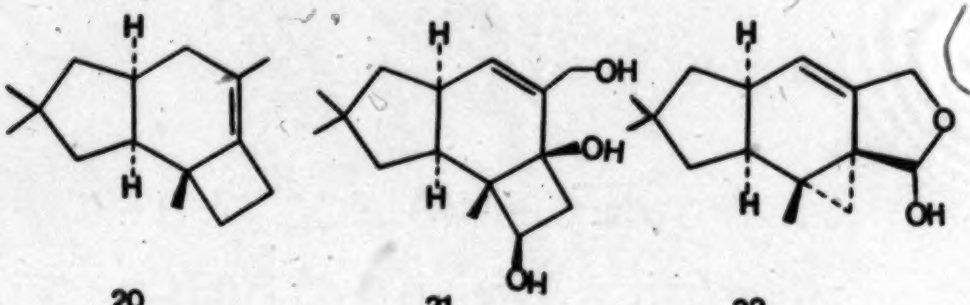


12 19

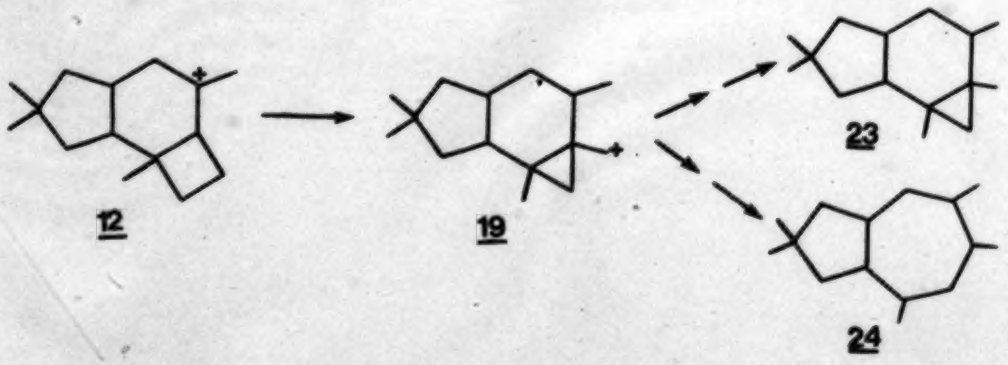
Further indirect evidence indicating the intermediacy of a protoilludane cation in marasmic acid biosynthesis was provided by Nozoe et al.[16] A study of the metabolites of the fungus Fomitopsis insularis by these authors shows that Δ^6-protoilludane (20) and neoilludol (21) co-occur with compound 22 which bears the skeleton of marasmic acid.



20 21 22

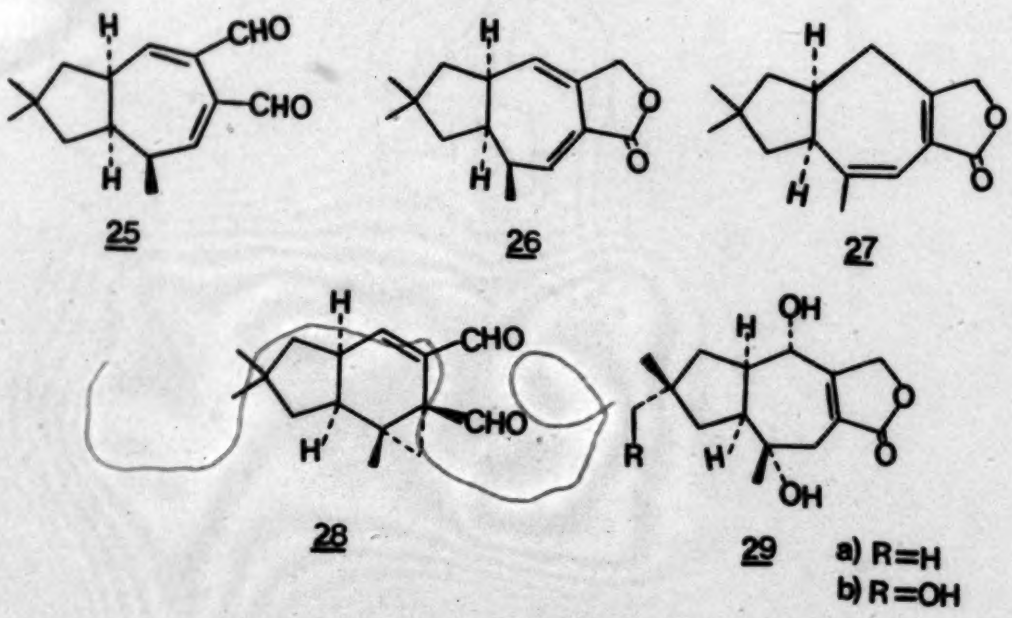
Since the discovery of marasmic acid (17) and its congeners, several sesquiterpenoids with novel skeletons have been isolated from fungal sources the biogenesis of which has been suggested to involve ring cleavage of the marasmate intermediate (19) derived by rearrangement of a protoilludane precursor (12).

The co-occurrence of metabolites with marasmane (23) and lactarane (24) skeletons have prompted several authors to suggest that these compounds are biogenetically derived from a common marasmate intermediate (19) (which in turn is derived from the protoilludane cation (12)).



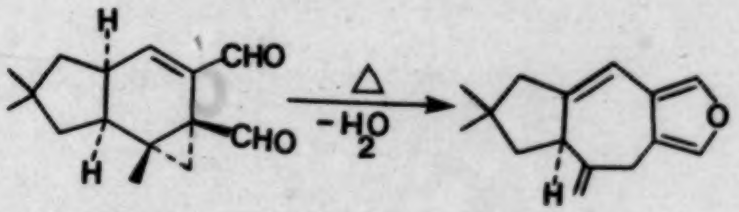
12 19

Velleral (25) and the two lactones 26 and 27, which all have the lactarane carbon skeleton, were isolated from the fungi Lactarius vellereus and L. pergamenus. Further examination of the above fungi revealed that isovelleral (28) which has the marasmane carbon skeleton is also a natural constituent of L. vellereus and L. pergamenus. Other examples of natural products possessing the lactarane skeleton (24) are lactarorufin A (29a) and lactarorufin B (29b), both isolated from the mushroom Lactarius rufus.[18,19]

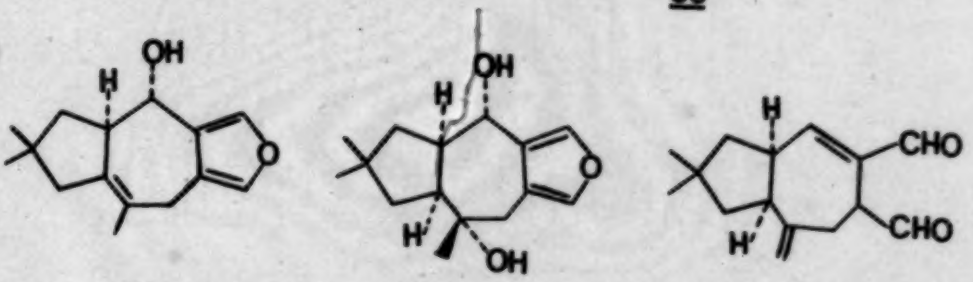


Magnusson et al. provided experimental support for the biogenetic interrelation between the marasmane and lactarane sesquiterpenoids.[20] Isovelleral (28) under-

went a smooth thermal rearrangement to the dienofuran 30 which bears the lactarane skeleton. These authors suggest that compound 30 is a reasonable biosynthetic precursor to the furan alcohols 31 and 32. Furthermore, an intermediate in this reaction, compound 33, may be a precursor to velleral (25).



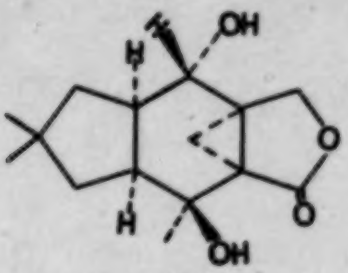
28 30



31 32 33

Isolactarorufin (34) is a novel tetracyclic sesquiterpene lactone co-occurring with lactarorufin A (29a) and lactarorufin B (29b) in the mushroom Lactarius rufus. The structure of 34 was determined by Daniewski and co-workers.[21] These authors proposed two biogentic pathways, both of which proceed from the protoilludane cation (12) via the tricyclic cation 35. However, path b was eliminated on the grounds

that it involved the intermediacy of cyclobutane cation 36 (sterpurane cation) which was an unknown skeleton at that time.



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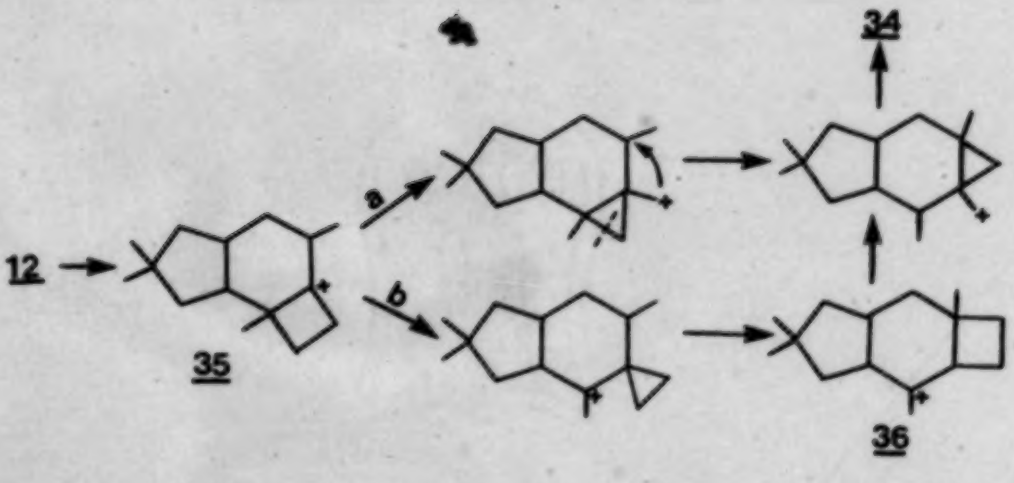
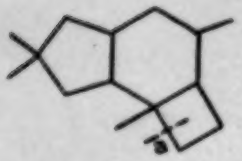


A variety of sesquiterpenoids have skeletons which are probably derived in nature by cleavage of proto-illudane (37) or illudane (38) precursors. Thus, cleavage of bond a in 37 or 38 would lead to the formation of compounds with the illudalane skeleton (39), and cleavage of bond b in illudane (38) would afford compounds with seco-illudane structure (40). Only one compound with the seco-illudane skeleton, hypacrone

(40a), is known.[22] Compound 40a is the acrid principle of a fern and its unique structure was confirmed by total synthesis.[23]



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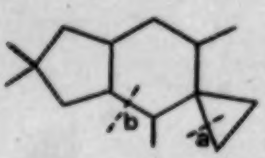

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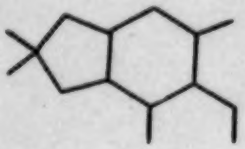

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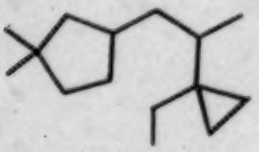

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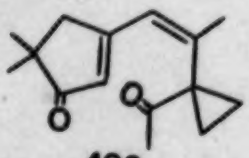

40a

Although the isolation of illudalic acid (41) as a "fourth crystalline compound"[24] accompanying illudin-M (10a) was first described in 1952 its structure, along with that of illudinine (42), was not established until 1969.[25] Like other members of this family of metabolites, these compounds incorporated [2-^{14}C] MVA. The subsequent isolation and structural determination of illudacetalic acid* (43) from the same source provided further evidence for the intermediacy of protoilludane (12) in the biosynthesis of metabolites with the illudalane skeleton.[26]

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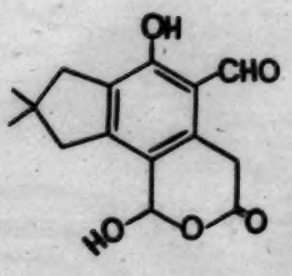
* Structure 43 is the revised structure reported by Woodward and Hoye[27] on the basis of synthetic studies.



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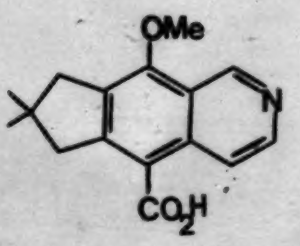

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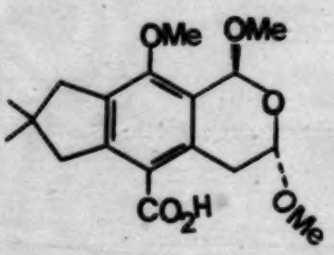
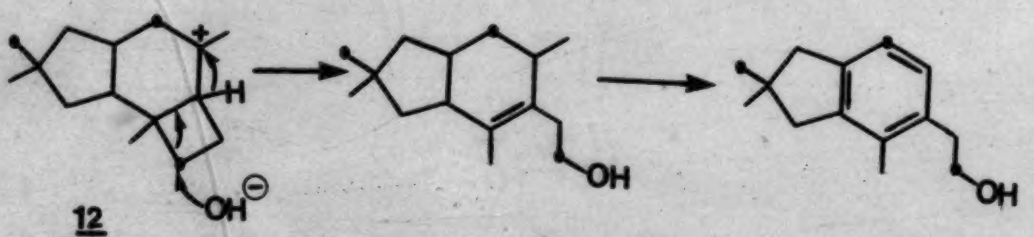


43

During the past decade a great number of pterosins and pterosides which bear the illudalane skeleton have been isolated from various species of fern.[28] The incorporation of [2-^{14}C] MVA into these compounds (e.g. pteroside A, 44) has provided evidence for the intermediacy of the protoilludane cation (12) in the biosynthesis of compounds with the illudalane skeleton.[29]



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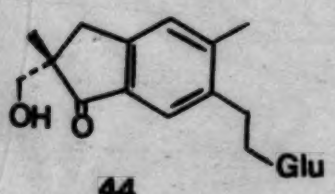
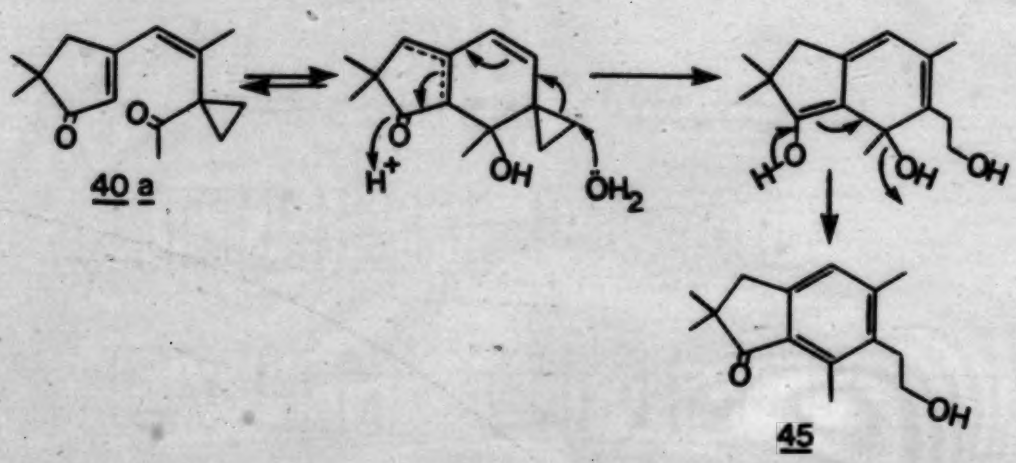


44

It is also interesting to note that treatment of hypacrone (40a) with acid provides a good yield of pterosin Z (45), providing experimental evidence that

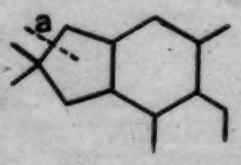
seco-illudanes may be in vivo precursors of illudalanes.[22]



40 a

45

Recently, Ayer and McCaskill in these laboratories have investigated the metabolites of the bird's nest fungus Cyathus bulleri.[30] These authors have characterized a series of sesquiterpenoid compounds, cybrodins, that represent a new class of compounds biogenetically obtained from illudalane (39) by bond cleavage at a. The major metabolite of C. bulleri is cybrodol (46).



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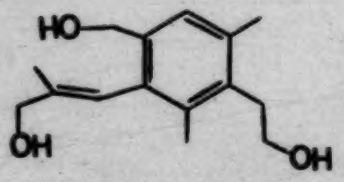
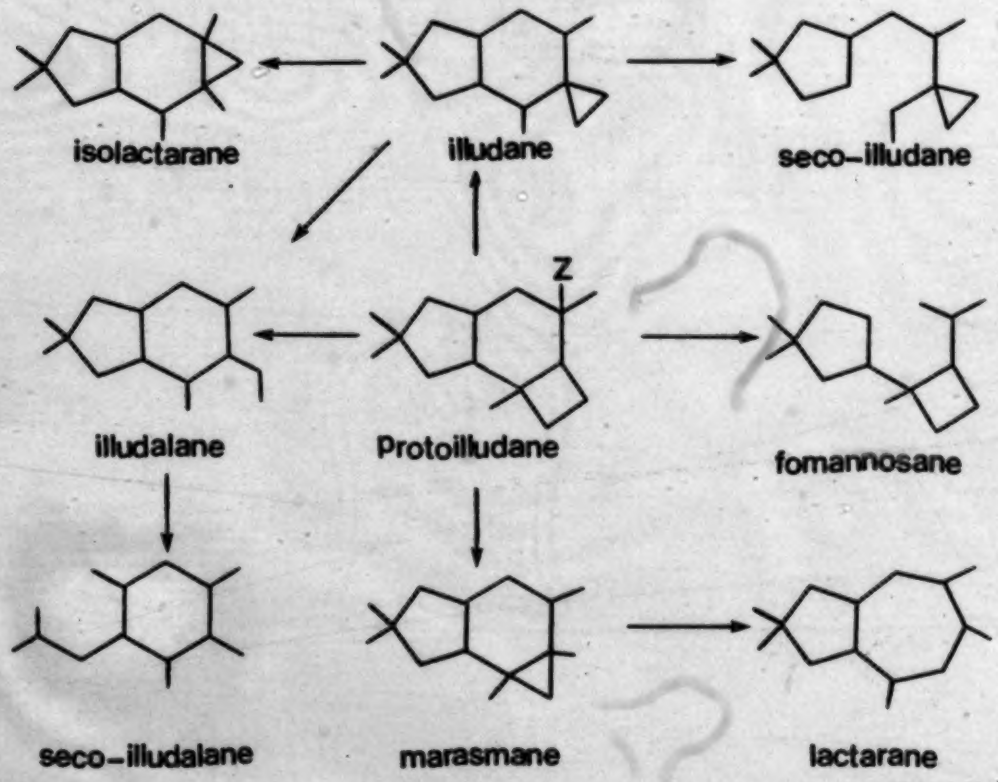


46

A schematic representation of the postulated involvement of the protoilludane intermediate in the biosynthesis of various sesquiterpenoid structures is outlined in the following scheme. It is interesting

to note that the fungus Fomitopsis insularis is capable of producing metabolites possessing the skeletons of several of these intermediates, namely, protoilludane, marasmane, lactarane, illudalane, and fomanosane.[16]

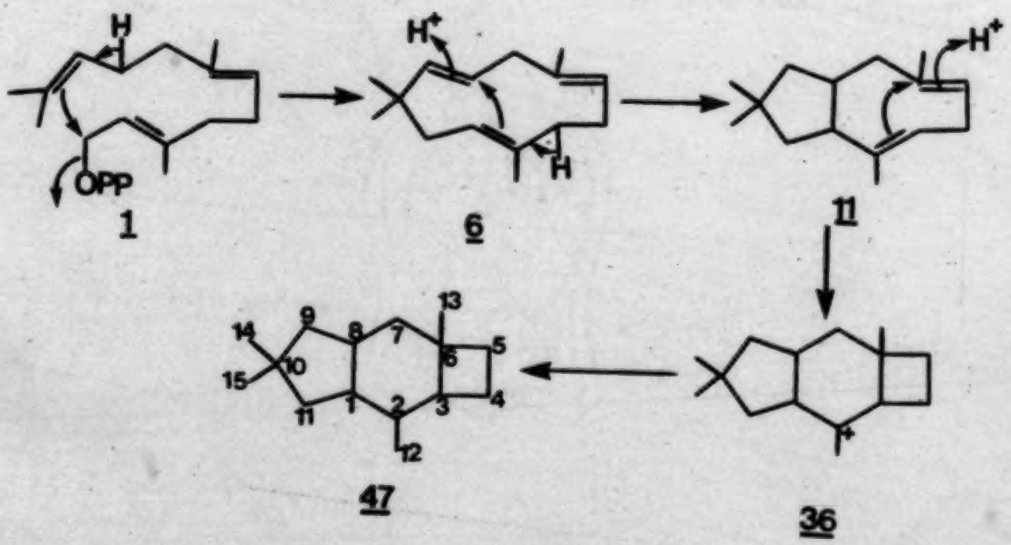


The purpose of the work reported in the part II of this thesis is to study the biosynthetic route leading to the sterpurane skeleton, which represents a completely new skeleton among the sesquiterpenoid metabolites. Stereum purpureum was grown in liquid culture and

[1-^{13}C]- and [2-^{13}C] acetate were incorporated into sterpuric acid and sterpurene-3, 12,14-triol to the extent of 5%. The ^{13}C nmr spectra of the enriched methyl sterpurate and the diacetate of sterpurene-3,12,14-triol were compared with the spectra of the same compounds containing natural abundance of carbon-13. This study provides evidence that the sterpurane skeleton is biogenetically derived from the protoilludane cation (12).

DISCUSSION

In the first part of this thesis it was shown that the sterpurane skeleton (47) can be derived from farnesyl pyrophosphate (1) via humulene (6), the [6.3.0]-bicyclic system 11, and the sterpurane cation (36). This biosynthetic proposal which helped in deriving the structure of sterpuric acid involves no rearrangement and is outlined in Scheme I.



SCHEME I

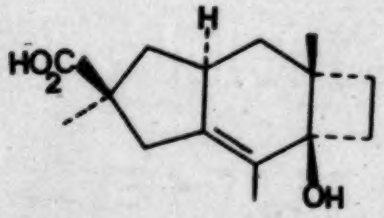
The above proposal seemed to be the most logical and straightforward route for the biosynthesis of sterpuric acid and its congeners. However, since these metabolites represent a unique carbon skeleton among the sesquiterpenoids, it seemed appropriate to examine the proposed

biogenesis. Therefore, we decided to undertake a study of the incorporation of ^{13}C-labelled acetate into sterpuric acid and sterpurene-3,12,14-triol. An analysis of the ^{13}C nmr spectra of these enriched metabolites would then provide information as to the biosynthetic pathway leading to the sterpurane skeleton.

S. purpureum was grown in malt extract-dextrose-peptone liquid culture. This culture was treated with sodium [1-^{13}C]- or [2-^{13}C] acetate using a modified procedure as described for the ^{13}C nmr studies on the biosynthesis of the cyathins.[31] Thus, after an initial 10 day growth period, the liquid culture of S. purpureum was treated with sodium acetate enriched with carbon-13 at either C-1 or C-2 (90% enriched)at a dosage of 1 mmole/ℓ. The feeding with enriched acetate was repeated twice more after 17 and 24 days growth. It should be pointed out that the advantage of multiple dosage over a single dose feeding lies in the opportunity it provides for maintaining a more or less steady concentration of the precursor while avoiding the possible toxic effects of an excessively high concentration of acetate.

After 30 days growth the mycelia were harvested and the crude extract containing metabolites enriched with ^{13}C were obtained by the usual work up. Sterpuric acid (48) enriched with [1-^{13}C] acetate was isolated

from the crude acidic mixture as described in Part I. It was then transformed into spectroscopically pure methyl sterpurate (49) by treatment with diazomethane followed by chromatography.



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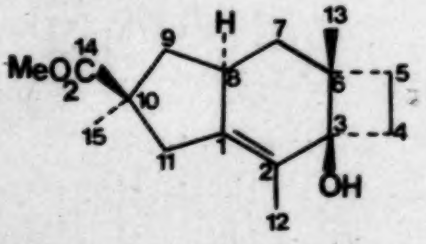


49

The proton noise decoupled (PND) ^{13}C nmr spectrum of 49 at natural abundance exhibits 16 signals (see also Part I). In order to follow the labelling pattern of methyl sterpurate, it was first necessary to assign the chemical shift of each carbon atom in the molecule of methyl sterpurate. The assignments of the individual carbon atoms are based largely on off-resonance and single frequency decoupling (selective proton decoupling) experiments.[32] The off-resonance decoupled spectrum may be used to determine which carbons and hydrogens are bonded, indicating whether the carbon atom in question is attached to 3, 2, 1, or no hydrogens. Single frequency decoupling experiments (observe ^{13}C, irradiate $\{^1H\}$) are utilized to establish which carbon is directly bonded to a particular proton. Therefore, an unambiguous assignment of the ^1H nmr spectrum of the compound

under investigation often provides the necessary tool to locate the majority of the carbon atoms within the molecule. The assignments of the protons in the ^1H nmr spectrum of sterpuric acid and its derivatives were dealt with in detail in the Part I of this thesis. The chemical shifts, splitting patterns, coupling constants and locations of all protons of methyl sterpurate (49) can be summarized in the following table.

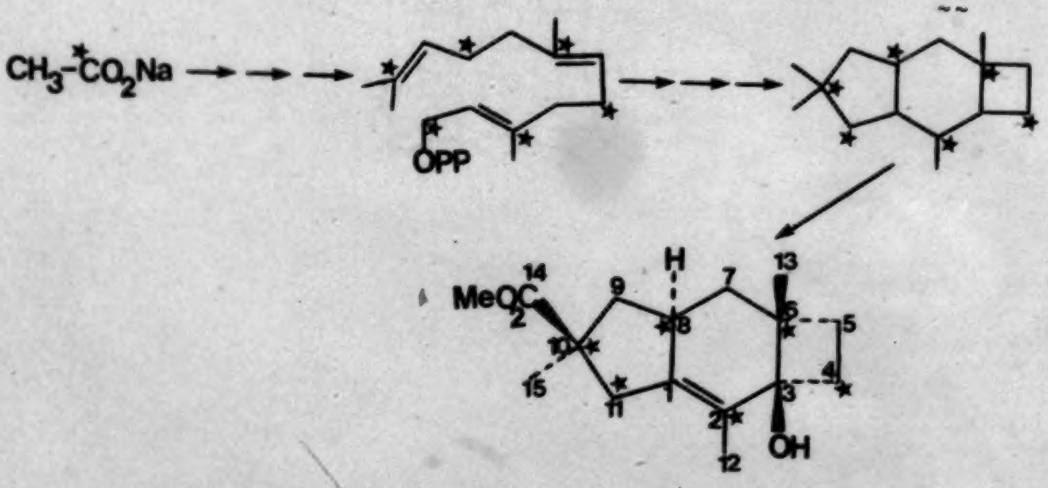
δ(ppm)	No. of H	splitting	J('s)(Hz)	location of H('s)
3.70	3	s	-	OCH_3
2.83	1	d	17	C-11
2.54	1	br. mult.	-	C-8
2.23	1	d	17	C-11
2.12	1	mult.	-	C-4
1.98	1	mut.	-	C-4
1.83	1	dd	7,12	C-9
1.64	1	dd	11,12	C-9
1.60	3	s	-	C-12
1.57	1	dd	6,13	C-7
1.49	1	mult.	-	C-5
1.34	3	s	-	C-15
1.23	1	mult.	-	C-5
1.20	3	s	-	C-13
0.87	1	dd	11,13	C-7

The utilization of the ^1H nmr data in selective proton

decoupling experiments along with the information deduced from PND- and off-resonance decoupled ^{13}C nmr spectra afforded the assignments of carbon atoms bearing hydrogen(s) in methyl sterpurate (49). The assignments of the sp^2 centers and the quaternary carbon atoms were obtained by comparison of the chemical shifts of these centers with similar cases reported in the literature.[33] Figure 1 shows the PND ^{13}C nmr spectrum of methyl sterpurate at natural abundance. The following table summarizes the chemical shifts, multiplicities, and locations of all carbon atoms in methyl sterpurate (49).

δ(ppm)	multiplicity	location of C
178.6	s	C-14
138.3	s	C-1
127.8	s	C-2
73.4	s	C-3
51.9	s	O-$\underline{C}H_3$
47.1	s	C-10
44.3	t	C-9
43.9	s	C-6
41.3	dd	C-11
36.8	d	C-8
34.9	dd	C-7
34.4	t	C-4
25.4	q	C-15
23.4	q	C-13
22.1	t	C-5
12.8	q	C-12

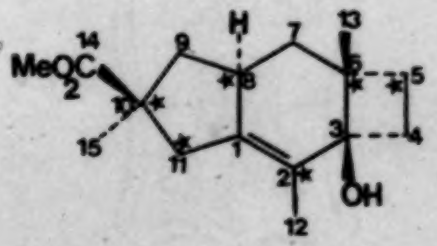
The PND ^{13}C nmr spectrum of methyl sterpurate enriched with [1-^{13}C] acetate was obtained under exactly the same conditions (the same temperature, concentration, and number of pulses applied). Comparison (Fig. 2) with that obtained for methyl sterpurate at natural abundance (Fig. 1) indicates that [1-^{13}C] acetate was incorporated into sterpuric acid to the extent of about 5%. Assuming that the pathway proposed for the biosynthesis of sterpurane skeleton (Scheme I) was correct, we expected to observe enhancement of peaks for C-2, C-4, C-6, C-8, C-10, and C-11 in the PND ^{13}C nmr spectrum of the enriched methyl sterpurate. Thus, it was assumed that C-1 enriched methyl sterpurate should have the labelling pattern shown in structure 50.



50

However, the ^{13}C nmr spectrum of methyl sterpurate enriched with C-1 labeled acetate does not exhibit enrichment at C-4. Instead, enrichment is observed

at C-5. Thus, the labelling pattern is not that shown in 50, but it is as shown in structure 51. Furthermore, if 50 represented the correct labelling pattern, one would expect to observe induced coupling only between C-10 and C-11 of the enriched methyl sterpurate. However, the analysis of the ^{13}C nmr spectrum of enriched methyl sterpurate revealed the existence of two such induced couplings, one due to the expected C_{10}-C_{11} coupling ($J_{C_{10}-C_{11}}$ = 34.3 Hz), and another due to C_5-C_6 coupling ($J_{C_5-C_6}$ = 29.3 Hz). The observation of this second induced coupling provided further evidence for the labelling pattern shown in 51.

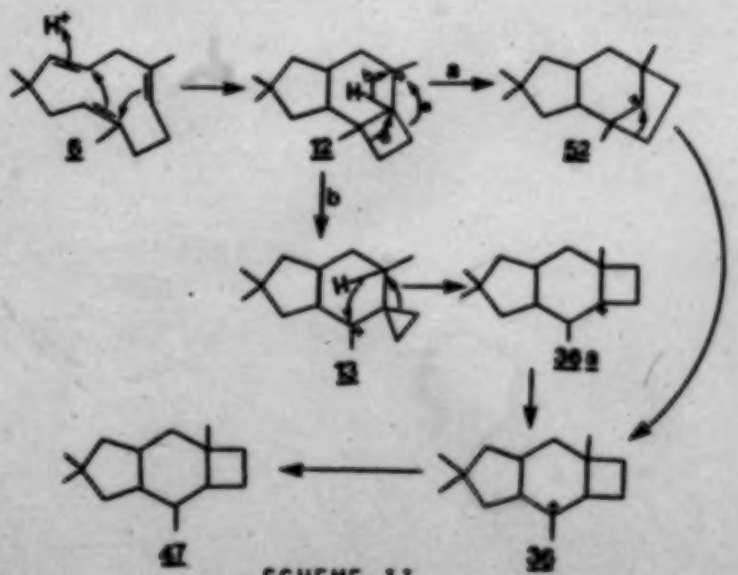


51

The results of the incorporation of [1-^{13}C] acetate into sterpuric acid showed that the pathway proposed for the biogenesis of sterpurane skeleton (Scheme I) was not followed. It was now obvious that the formation of the cyclobutane portion of the sterpurane skeleton did not involve a simple cyclization of 11 to afford the sterpurane cation (36).

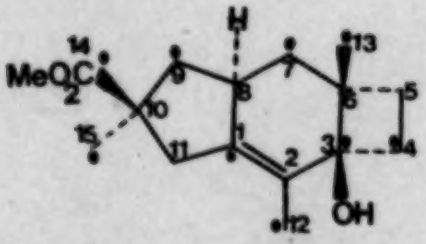
The precedence of the involvement of the protoilludane

cation (12) in the biosynthesis of serveral novel skeletons isolated from different species of Basidiomycetes prompted us to consider the following biosynthetic pathway (Scheme II). As in the first proposal, it starts with initial cyclization of farnesyl pyrophosphate to humulene (6). The latter then reacts further to afford the protoilludane cation (12). The cation 12 may then rearrange to the bicyclo[3.2.1]octane system 52. A further 1,2-alkyl rearrangement in 52 leads to the sterpurane cation which is then further transformed to sterpurane (47) (path a). Alternatively, the protoilludane cation (12) can rearrange to the known illudane cation (13). This may then undergo a 1,3-hydride shift followed by a 1,2-alkyl rearrangement to provide the tricyclic cation 36a which may lead to the sterpurane cation (36) (path b).



SCHEME II

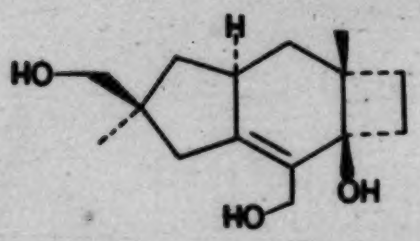
At this point, it was obvious that if the biosynthetic route proposed in Scheme II was correct, then the ^{13}C nmr spectrum of methyl sterpurate derived from [2-^{13}C] acetate would have enrichment of the ring carbon atoms at C-1, C-3, C-4, C-7, and C-9 as well as the three methyl groups and the carboxyl carbon of methyl sterpurate as shown in 53.



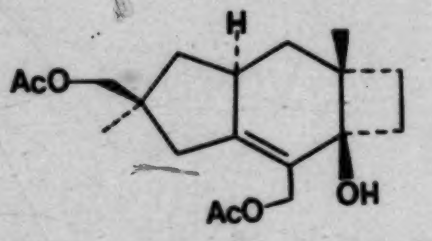
53

To our disappointment, when S. purpureum was grown in the presence of sodium [2-^{13}C] acetate it did not produce a sufficient quantity of enriched sterpuric acid for ^{13}C nmr studies. Sodium acetate enriched with carbon-13 is relatively expensive. Also, there was no guarantee that repeating the experiment would provide a sufficient amount of sterpuric acid. We thus decided to examine the sterpurene-3,12,14-triol (54) formed in these experiments. Compound 54 is the major component of the neutral portion of the culture broth extract. The structural elucidation of this compound is described in Part I of the thesis. In order to obtain a sufficient quantity of pure sample and also to circumvent the problem of dissolving 54 in

a small volume of $CDCl_3$, the triol 54 was converted to the corresponding diacetate 55.



54



55

The spectral properties of 12,14-O,O-diacetylsterpurene-3,12,14-triol (55) were discussed in Part I. However, it was now necessary to assign completely the ^1H nmr spectrum of 55. This would provide the necessary means for selective proton decoupling experiments to be used for assignment of all the carbon atoms in the ^{13}C nmr spectrum of 55. The following table shows the assignment of all protons in the ^1H nmr of the diacetate 54 as verified by double irradiation experiments.

δ(ppm)	No. of H	splitting	J('s)(Hz)	location of H('s)
4.74	2	ABq	12	C-12
3.90	2	ABq	12	C-12
2.68	1	br. mult.	-	C-8
2.42	1	d	17	C-11
2.25	1	d	17	C-11
2.20	1	mult.	-	C-4
2.08	3	s	-	$CH_3C=O$

continued.....

δ(ppm)	No. of H	splitting	J('s)(Hz)	location of H('s)
2.07	1	mult.	-	C-4
2.06	3	s	-	$\underline{CH_3}C=O$
1.71	1	dd	12,12.5	C-9
1.63	1	dd	6,13	C-7
1.55	1	mult.	-	C-5
1.30	1	mult.	-	C-5
1.26	1	dd	6,12.5	C-9
1.24	3	s	-	C-13
1.14	3	s	-	C-15
0.88	1	dd	11,13	C-7

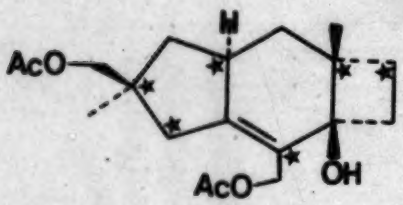
The PND ^{13}C nmr spectrum of the diacetate 55 shows the presence of 19 carbon atoms. Selective proton decoupling experiments provided the assignment of all carbon atoms bearing hydrogen(s). Again, the assignment of the carbon atoms of the tetrasubstituted double bond and the quaternary carbon atoms was made by comparison to similar cases reported in the literature.[33] These results are summarized in the following table.

δ(ppm)	multiplicity	location of C
171.2	s	2 x C=O
147.6	s	C-1
127.2	s	C-2
72.9	s	C-3
72.4	t	C-14
62.1	t	C-12
44.2	s	C-6
42.9	dd	C-9

continued.....

δ(ppm)	multiplicity	location of C
40.6	s	C-10
40.0	t	C-11
37.1	d	C-8
35.3	t	C-4
35.2	dd	C-7
24.9	q	C-15
23.9	q	C-13
22.1	t	C-5
21.0	q	$\underline{C}H_3C=O$
20.9	q	$\underline{C}H_3C=O$

The ^{13}C nmr spectrum of 12,14-O,O-diacetylsterpurene-3,12,14-triol prepared from C-1 labeled acetate (56) (Fig. 3) exhibits enrichment at C-2, C-5, C-6, C-8, C-10, and C-11. This is in agreement with the biosynthetic pathway outlined on Scheme II. The spectrum also exhibits induced couplings between C-5 and C-6 ($J_{C_5-C_6}$ = 29.1 Hz) and C-10 and C-11 ($J_{C_{10}-C_{11}}$ = 34.3 Hz). The ^{13}C nmr spectrum of the same compound prepared from C-2 labeled acetate (57) (Fig. 4) is also in complete agreement with the proposed biosynthesis. It shows the expected enrichment of the ring carbon atoms at C-1, C-3, C-4, C-7, and C-9 as well as the two carbon atoms bearing acetate functions (C-12 and C-14) and methyl groups (C-13 and C-15). Significantly, it also exhibits induced coupling between C-3 and C-4 ($J_{C_3-C_4}$ = 31.4 Hz).



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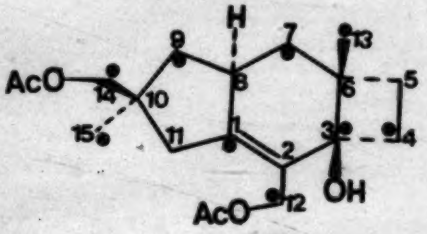
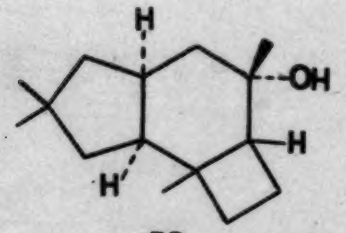


57

The ^{13}C nmr studies of methyl sterpurate and 12,14-O,O-diacetylsterpurene-3,12,14-triol enriched by means of [1-^{13}C]- or [2-^{13}C] acetate provide information on the biosynthesis of the sterpurane skeleton. These studies suggest that the biogenesis of this skeleton occurs via humulene (6), the protoilludane cation (12), and either the illudane cation (13) or the cation 52 (Scheme II). However, the above labelling studies do not differentiate between the cations 13 or 52 as an intermediate in the course of biosynthesis. The illudane cation (13) is known to be an important precursor in the biogenesis of a variety of fungal metabolites. To the best of the author's knowledge, the cation 52 has not been reported as an intermediate in the biosynthesis of any sesquiterpenoid metabolites. It is interesting to note, however, that the chemically generated protoilludane cation is transformed into a system with the carbon skeleton of 52.[34] Thus, treatmetn of the synthetic protoilludane derivative 58 with formic acid resulted in rearrangement to 59. This,

along with the fact that treatment of methyl 1,2-epoxy-sterpurate (20, Part I) afforded a compound (21, Part I) with the carbon skeleton of 52, provides some experimental support for the possible intermediacy of the cation 52 in the biosynthesis of sterpurane skeleton (47).



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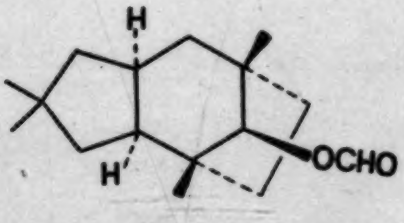

59

EXPERIMENTAL

The reader is referred to the Experimental Section of Part I of the thesis for general information about chemicals, solvents, chromatographic materials, and spectroscopic instruments utilized for this part of the study. Details concerning the isolation and structural determination of sterpuric acid (48) and sterpurene-3,12,14-triol (54) may also be found in the Experimental Section of Part I. The procedures for conversion of sterpuric acid (48) to methyl sterpurate (49) and transformation of the triol (54) to 12,14-O,O-diacetylsterpurene-3,12,14-triol (55) and the spectral properties of these derivatives are also described in Part I.

The ^{1}H nmr spectra and ^{13}C nmr spectra of methyl sterpurate at natural abundance (49) and enriched (51) were measured on a Bruker WH-400 spectrometer. The same instrument was used to measure the ^{1}H nmr and ^{13}C nmr spectra of 12,14-O,O-diacetylsterpurene-3,12,14-triol at natural abundance (55) as well as the enriched (56 and 57) samples. Samples of methyl sterpurate were examined as 3.6% (W/V) solutions in $CDCl_3$ and the samples of 12,14-O,O-diacetylsterpurene-3,12,14-triol were examined as 5.6% (W/V) solutions in the same solvent. In all cases TMS was used as an internal

reference. The precision of ^{13}C shielding data is judged to be ± 0.004 ppm. Sodium [1-^{13}C] acetate and sodium [2-^{13}C] acetate (90% enriched) were purchased from Merck, Sharp and Dohme Canada Ltd.

Incorporation of ^{13}C-labeled acetate into sterpuric acid and sterpurene-3,12,14-triol

Stereum purpureum (C-663) was grown in liquid still culture at room temperature in malt extract (25 g/ℓ) - dextrose (13 g/ℓ) - peptone (0.7 g/ℓ) medium in ten 2.8 ℓ Fernback flasks (1 liter medium per flask). After 10 days growth sodium [1-^{13}C] acetate (82 mg, 1.00 mmole) dissolved in water (5 ml, sterilized) was injected into each flask. One week later an additional 1.00 mmole sodium [1-^{13}C] acetate was added to each flask. In another week a further 1.00 mmole sodium [1-^{13}C] acetate was added to each flask. After a total of 30 days growth the mycelium was removed and the broth was extracted as described in Part I. The labeled sterpuric acid and sterpurene-3,12,14-triol were isolated and identified by the same procedures used for their natural abundance counterparts. The enriched sterpuric acid and sterpurene-3,12,14-triol were converted to the labeled methyl sterpurate (18 mg, 51) and 12,14-O,O-diacetylsterpurene-3,12,14-triol (28 mg, 56) by the same procedures used for the preparation

of their natural counterparts.

The experiments with sodium [2-^{13}C] acetate were conducted in exactly the same way as described for sodium [1-^{13}C] acetate to afford C-2 labeled 12,14-*O*,*O*'-diacetylsterpurene-3,12,14-triol (28 mg, **57**).

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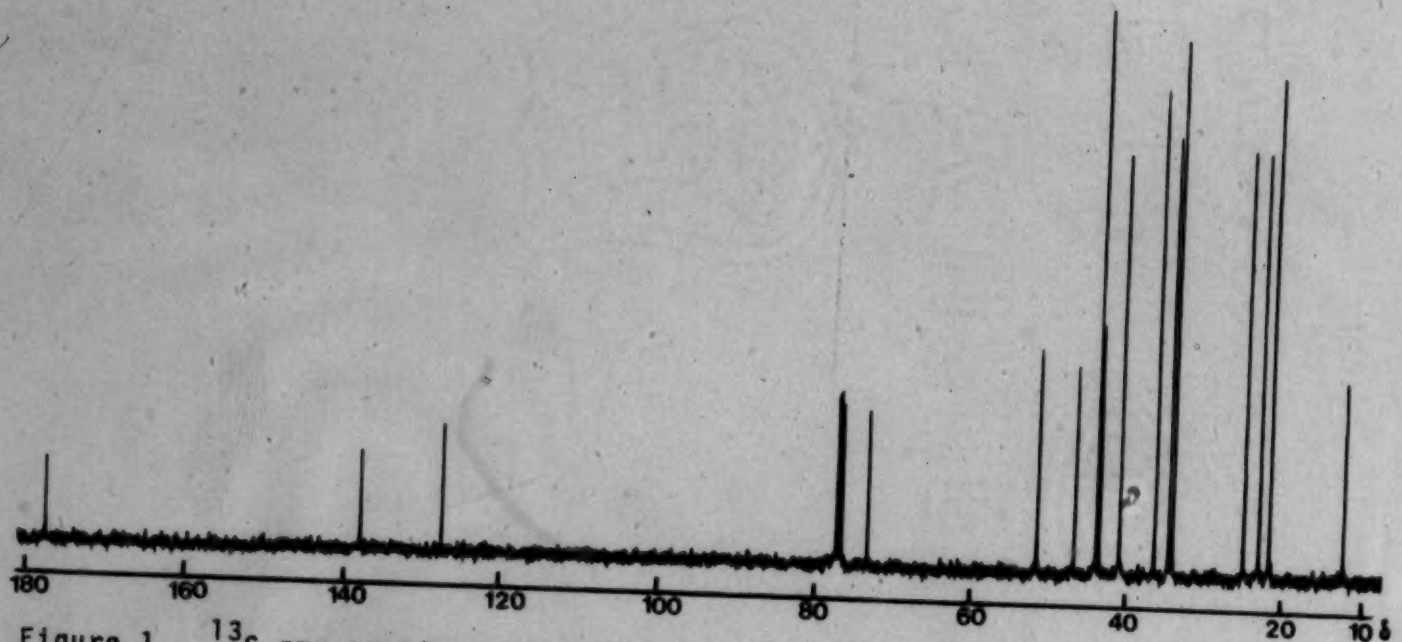

Figure 1. ^{13}C nmr spectrum of methyl sterpurate at natural abundance (49).

APPENDIX

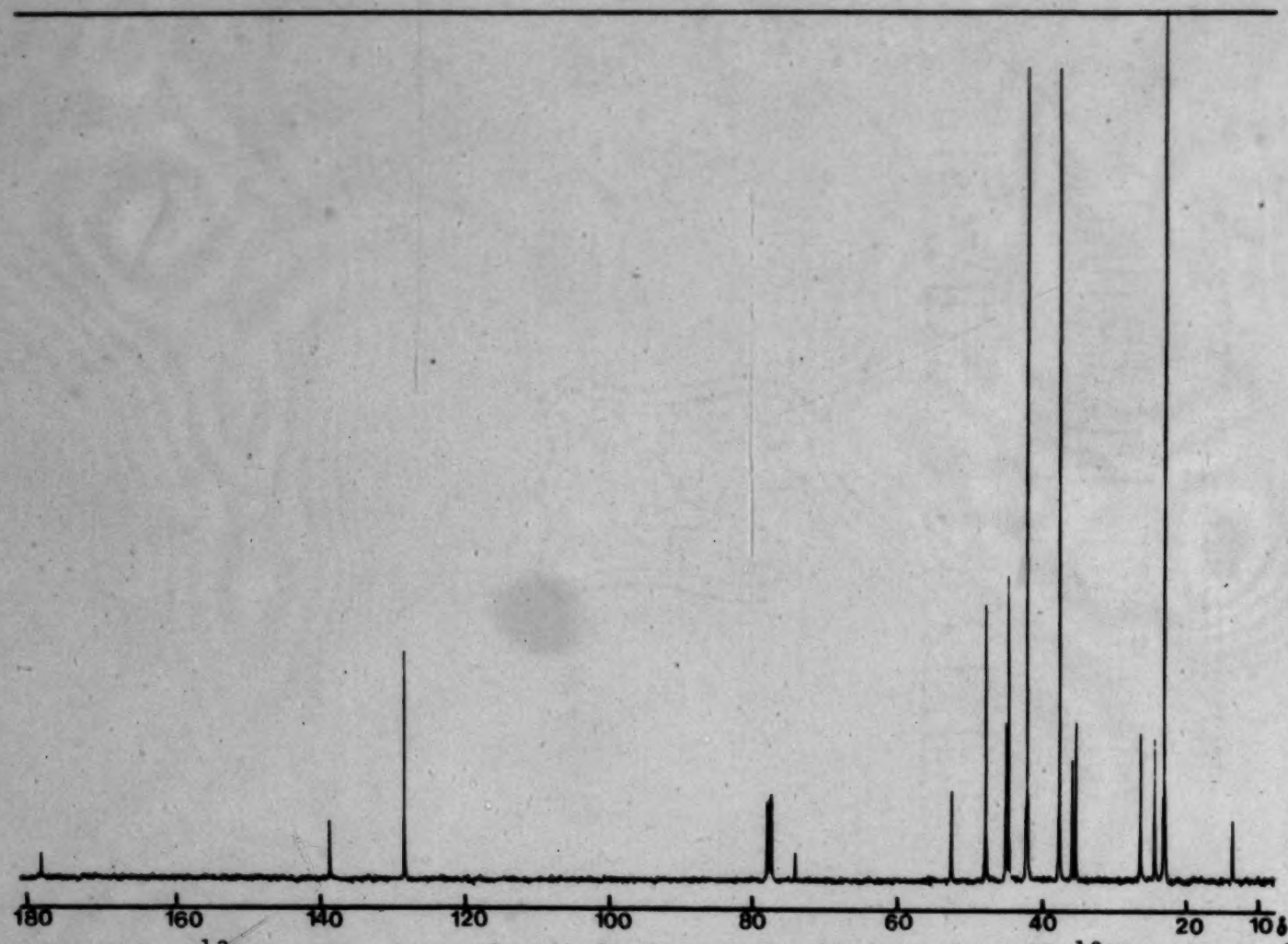

Figure 2. ^{13}C nmr spectrum of methyl sterpurate obtained from [1-^{13}C] acetate (51).

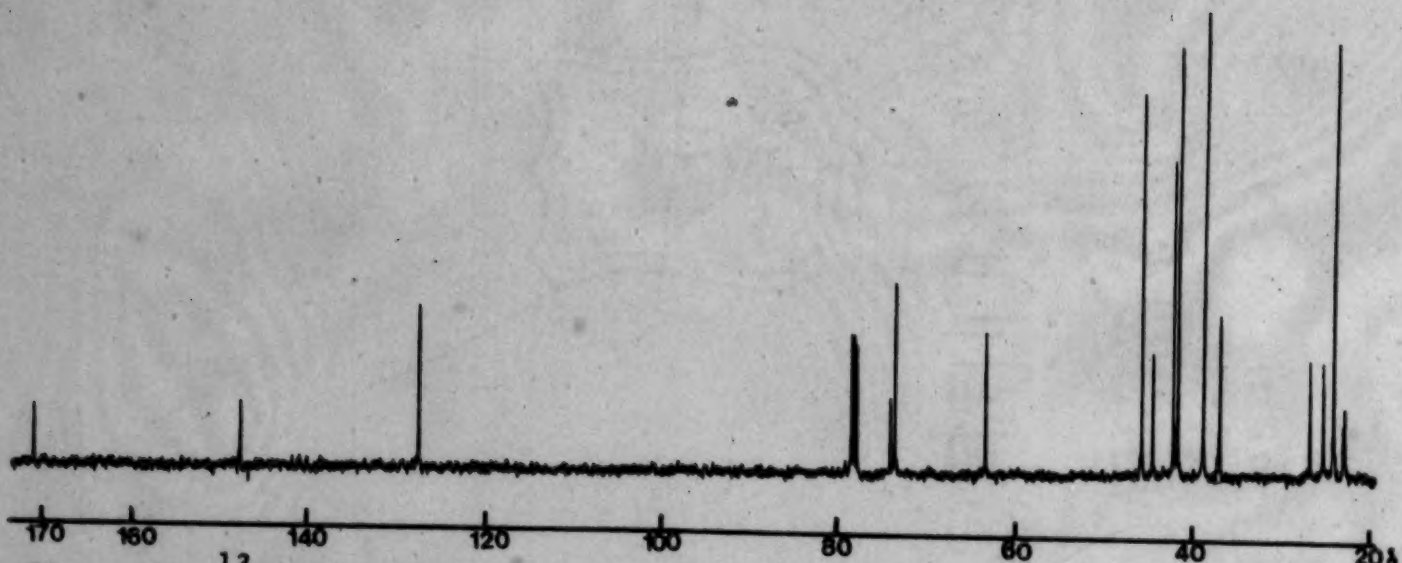

Figure 3. ^{13}C nmr spectrum of 12,14-O,O-diacetylsterpurene-3,12,14-triol obtained from [1-^{13}C] acetate (56).

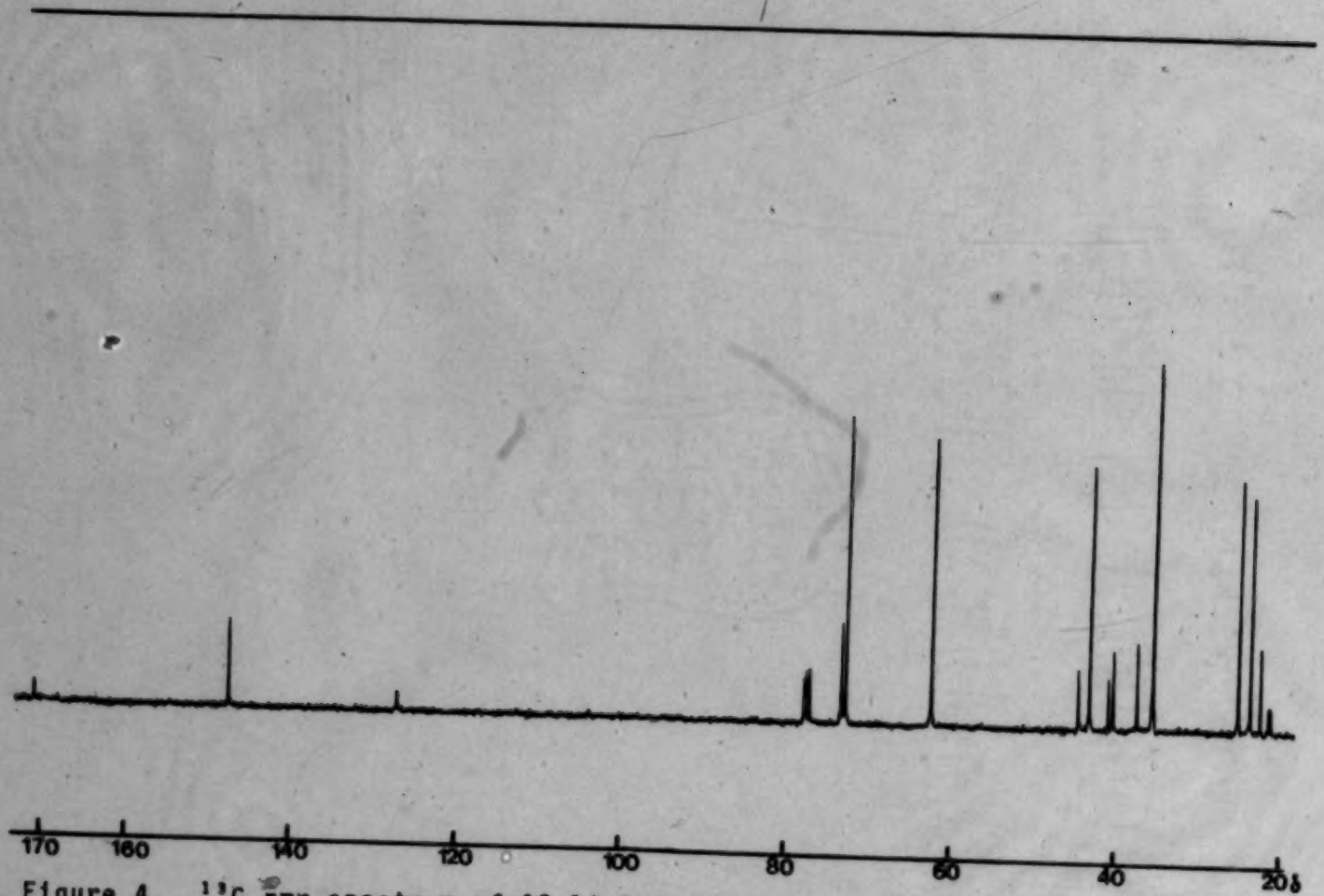

Figure 4. ^{13}C nmr spectrum of 12,14-O,O-diacetylsterpurene-3,12-14-triol obtained from [2-^{13}C] acetate (57).